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THE THEORETICAL RESPONSE OF LIVING CELLS TO CONTACT WITH SOLID BODIES.

By WALLACE O. FENN.

(*From the Laboratory of Applied Physiology, Harvard Medical School, Boston.*)

(Received for publication, December 23, 1921.)

Recent discussions of the behavior of living cells in contact with solid bodies have been published by Loeb (1) and Tait (2). Both authors admit that two factors are involved in the reactions of the cells—changes in consistency of the protoplasm and surface tension forces. Tait lays particular stress on the latter, while Loeb believes that changes in consistency are more important. The latter has, perhaps, taken a somewhat safer position in his interpretation of the phenomena described. It would seem, however, that the relative importance of these two factors must vary with the particular type of cell chosen and the conditions of observation. Thus, invertebrate blood cells are more at the mercy of surface tension forces on coming into contact with a solid body than *Amœba* or mammalian leucocytes. It is certainly true, nevertheless, that all blood cells are subjected to the abnormal forces of surface tension when they strike a foreign body. Whether or not these forces are sufficient to determine the behavior of the cell depends upon its fluidity. If it is only to enable us to discuss intelligently the relative importance of consistency changes and surface tension forces as applied to cells, it is necessary to formulate these hypotheses as accurately as possible.

There is, unfortunately, little to be said about such an indefinite factor as changes in the consistency of protoplasm, but surface tension lends itself readily to quantitative treatment. Tait has endeavored to predict the behavior of cells in contact with flat surfaces and small solid bodies from principles of surface tension. His discussion, however, was both incomplete and, in respect to phagocytosis of small bodies, erroneous. It seems important, therefore, to amplify and correct his discussion of the theory.

To begin with a simple case, we may consider the various positions which a perfectly fluid hypothetical cell would assume on a flat surface of glass in terms of the surface tensions between the cell and the plasma, the plasma and the glass, and the glass and the cell. In Fig. 1 let *a* represent the cell suspended in plasma, *P*, before coming into contact with the glass, *G*, and let *b* represent the same cell in an equilibrium position with respect to *G*. In taking this position or any other position in contact with any solid body, *G*, the *cp* interface, *x*, has been increased (or decreased), and an area, *s*, of the *gp* inter-

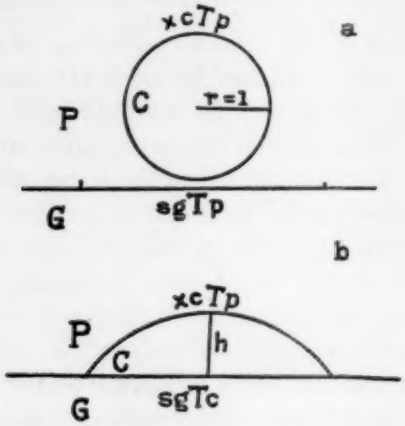

FIG. 1. Diagram of a liquid sphere, *C*, representing a cell suspended in plasma *P*, before (*a*) and after (*b*) coming into contact with a glass surface, *G*. *x* is the area of the *cp* interface; *cTp*, the surface tension of the *cp* interface; *s*, the area of the *gc* interface at (b) and the corresponding and equal *gp* interface at (*a*).

face has been exchanged for an equal area of the *gc* interface. The surface energy, *E*, at *b* is expressed by the equation

$$E = xcTp + sgTc - sgTp \qquad (1)$$

where *cTp*, *gTc*, and *gTp* represent the surface tensions of the *cp*, *gc*, and *gp* interfaces respectively.

The problem is to calculate the height, *h*, of the cell above the glass at equilibrium in terms of *gTc*, *gTp*, and *cTp*, the volume of the cell remaining constant. Now by definition the surface energy, *E*, at equilibrium must be at a minimum, and $\frac{dE}{dh} = 0$. To find $\frac{dE}{dh}$ let us

express x and s in equation (1) in terms of h when the radius, r, of the cell (when spherical as at a) is equal to 1. In order to do this it is necessary to neglect the effect of gravity and assume that C always has the shape of a sphere or spherical segment. This is quite legitimate since the effect of gravity would be practically negligible where the dimensions of C are those of leucocytes and where the difference in density between C and P is small.[1] By familiar formulas we may write

$$x = \pi (a^2 + h^2) \tag{2}$$

$$s = \pi a^2 \tag{3}$$

where a is the radius of the base of the cell.

The volume of the spherical segment at b equals the volume of the sphere at a (Fig. 1) or

$$\frac{1}{6}\pi h (h^2 + 3a^2) = \frac{4}{3}\pi r^3$$

Putting $r = 1$ and solving

$$a = \sqrt{\frac{8}{3h} - \frac{h^2}{3}} \tag{4}$$

Substituting the value of a from equation (4) in (2) and (3) and introducing the resulting values of x and s in equation (1) we have

$$E = cTp\left(\pi h^2 + \frac{8\pi}{3h} - \frac{h^2\pi}{3}\right) + (gTc - gTp)\left(\frac{8\pi}{3h} - \frac{\pi h^2}{3}\right) \tag{5}$$

Putting $n = cTp$ and $m = gTc - gTp$, differentiating with respect to h, and simplifying, we find

$$\frac{dE}{dh} = \frac{\pi h^3\left(2n - \frac{2}{3}(m+n)\right) - \frac{8\pi}{3}(m+n)}{3h^2} \tag{6}$$

At equilibrium $\frac{dE}{dh} = 0$. Hence, putting the right hand member of equation (6) equal to 0 and solving for h, we have

[1] Gravity would merely shift the equilibrium point without altering the principles involved.

$$h = \sqrt[3]{\frac{8\ (m+n)}{4n-2m}} = \sqrt[3]{\frac{8\ (gTc - gTp + cTp)}{4cTp - 2gTc + 2gTp}} \tag{7}$$

With this equation, knowing m and n, we can calculate h in terms of the original radius of the cell and from h, in turn, we can calculate by equation (4) the radius of the base of the cell, a.

Another formula for the equilibrium is given by Tait from considerations of the contact angle in Fig. 2.

$$gTp = gTc + cTp \cos A \tag{8}$$

$$gTp - gTc = cTp \cos A$$

$$-m = n \cos A \tag{9}$$

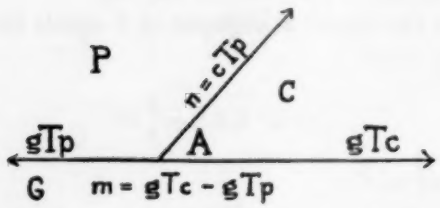

FIG. 2. Diagram of the equilibrium of surface tension forces at the angle of contact, A, between a hypothetical cell, C, suspended in plasma, P, and a glass slide, G. Arrows indicate direction of pull only.

By simple geometry and trigonometry it may be shown that

$$\cos A = \frac{4 - 2h^2}{4 + h^2} \tag{10}$$

Substituting the value of h from equation (7) in (10) and reducing, we have $\cos A = -\frac{m}{n}$ which is the same as equation (9), and *proves that the same equilibrium results either from considerations of the contact angle or considerations of the surface energy.*

Now Tait's argument is incomplete because in using equation (9) he assumes that $A = 0$, *i.e.*, that the cell spreads to infinity, whence $\cos A = 1$ and $m + n = 0$ or, in his terminology, a cell will spread to infinity[2] when

[2] The cell is, of course, assumed to be a mathmatical sphere without structure. Even a pure liquid could not spread out in a layer less than one molecule thick.

$$gTc - gTp + cTp = \text{or} < 0$$

But equation (7) as well as equation (9) proves that a cell may spread without spreading to infinity; indeed, any position is possible. Thus in equation (7) if $n = +1$ and $m = +1$, then $h = 2$, *i.e.*, twice the radius of the cell when spherical, and the cell will not spread at all on G. If $n = +1$ and $m = -1$, then $h = 0$ and the cell will spread to infinity. If, however, to take an intermediate case, $n = +1$ and $m = 0$, then $h = \sqrt[3]{2} = 1.26$, which means that the cell will take the position of a hemisphere, having the same volume as the original sphere. At this point $\cos A = -\frac{m}{n} = 0$ and $A = 90°$.

When $m = 0$ there is neither gain nor loss of energy when an area of the interface between G and the plasma is replaced by an equal area of the interface between G and the cell. The explanation of the hemisphere as the equilibrium shape under these circumstances (when $m = 0$) is that in this position the surface of the cell exposed to the plasma is at a minimum. This brings out the significant fact that the area of the exposed surface of a liquid sphere of diameter, d, which is spreading to infinity on a flat surface, first decreases, passes through a minimum when the apparent diameter (diameter of the base) is $1.26d$, then increases until, at an apparent diameter of $1.86d$, it is again equal to the original surface area, and finally increases to infinity. Exactly comparable changes in the surface area of the cell occur during the ingestion of a small particle, except that the final increase is limited by the size of the particle instead of by infinity. This is clearly the reason why adhesiveness is such a familiar property of blood cells. We have thus been led to a definition of what we mean by adhesiveness or stickiness of cells. *A cell which is stuck to a slide is one that is incompletely spread out by forces of surface tension.* The energy necessary to detach the cell is stored up as surface energy on the newly formed surfaces. If the cell tears, leaving a layer of protoplasm still clinging on the slide, we have an exception in which the energy expended is merely a measure of the cohesion of the protoplasm (surface tension between protoplasm and protoplasm). It is of course possible that the natural rigidity of a cell will prevent it from spreading out on a solid surface so far and, therefore, from

sticking as hard as it otherwise would, but it is always true that the force which holds a cell to a glass slide, indeed which holds any liquid to any solid surface, is the force of surface tension.[3]

In extending this discussion to cover cases where the surface of G is curved and G becomes a small sphere, Tait makes a fundamental error. He argues that a particle of G will be ingested by C if the surface energy can thereby be decreased. In other words, G will be ingested if the decrease in energy, due to exchanging $sgTp$ for $sgTc$ ($-sm$), more than compensates for the increase in energy due to the enlargement of the cp interface (Δxn) after ingestion of G. Thus a particle will be ingested if $-sm > \Delta xn$ or if $sm + \Delta xn < 0$.

For the comparison with the condition necessary for the spreading of a cell on a flat surface, that $m + n =$ or < 0, Tait puts this inequality into the form $m + \frac{\Delta x}{s} n < 0$ and reasons that since $\frac{\Delta x}{s}$ is "as a rule"[4] less than 1, $m + \frac{\Delta x}{s} n$ will be more likely to be less than 0 than will $m + n$. Hence he predicts that if a cell ingests a small particle of G it will surely spread on a flat surface but that the reverse may or may not be true.

This prediction is erroneous, because, as we shall attempt to show, even though the surface energy may be less at complete ingestion than before ingestion, *it is always at a minimum (still less) at incomplete ingestion*, unless $m + n = 0$; *i.e.*, unless the cell would spread to infinity on a flat surface. This means that no particle of G can ever be completely ingested by C unless C will spread to infinity on G.

The truth of this statement becomes evident from considerations of the contact angle between C and G. As G becomes more and more nearly ingested the angle of contact approaches 0. It can never reach 0 unless $-\frac{m}{n} = 1$ for from equation (9)

[3] Some writers (Mathews, A. P., *Physiol. Rev.*, 1921, i, 553) would restrict the term surface tension to the free energy of cohesion on liquid surfaces. It is used here to denote the intensity factor of the free surface energy on either solid or liquid surfaces regardless of the nature of the forces involved.

[4] Actually it is always less than 1. It approaches 1 as a limit as the diameter of the ingested particle approaches infinity.

$$\cos A = -\frac{m}{n} = \cos 0 = 1$$

Since this subject has apparently never been worked out from the point of view of surface energy it seemed desirable to be satisfied that, when the forces of surface tension at the contact angle are in equilibrium, the surface energy is also at a minimum. Unfortunately, we have not been able, even with the expert assistance of a professional mathematician, to express x and s of equation (1) in terms of the degree of ingestion of a particle as measured by the length of the line y in Fig. 3, and thereby to obtain the value of $\frac{dE}{dy}$ and finally an equation like (7) for a surface of any degree of curvature. Theoretically this procedure is not impossible, but the necessary equations are too difficult to solve. A test case has been taken, however, where the radius of the particle, g, is one-quarter of the radius of the cell ($r = 1$) and the values of Δx (the change in surface area of the cell in contact with the plasma), and of s (the surface area of G in contact with C), have been calculated for the different values of y, the height of the spherical segment inside the cell (Fig. 3).[5] These values are given in Table I together with the cosine of the corresponding angle of contact. By assigning various values to m and n and using the calculated values of Δx and s in the equation[6] $sm + \Delta xn = E$, curves can be plotted showing how the surface energy, E, varies as y increases to 0.5 or twice the radius of the particle, G; *i.e.*, as ingestion approaches completion. These curves, for different values of m when $n = 1$, are shown in Fig. 4. The values of m used were calculated from the equation for the contact angle equilibrium, $m = -n \cos A$ where A is the angle of contact corresponding to the chosen value of y and n

[5] The complication arising from the increase in the radius of the cell to $r + \Delta r$, as the particle is more and more completely ingested is responsible for the difficulty of the calculation. In order to evaluate Δx and s it is necessary to solve for s (Fig. 3) in an equation involving z^3 and z^2 by trial and error. This must be done to four significant figures in order to calculate Δx and s accurately enough for the purpose.

[6] This equation is the same as (1), replacing gTc-gTp by m and cTp by n as in equation (6) and omitting the constant $4\ \pi r^2 n = 12.566\ n = (x - \Delta x)n$ representing the surface energy of the cell when spherical.

= 1. On each curve the point corresponding to the value of cos A (Table I) used in calculating m for that curve is marked by a circle.[7] The fact that the surface energy is always at a minimum at the marked point proves that, when the forces of surface tension are in equilibrium at the contact angle, the surface energy of the whole system is also at a minimum. That part of each curve beyond the minimum is dotted to indicate that it is imaginary, because the cell would continue to ingest a particle only as long as the surface energy was decreasing.

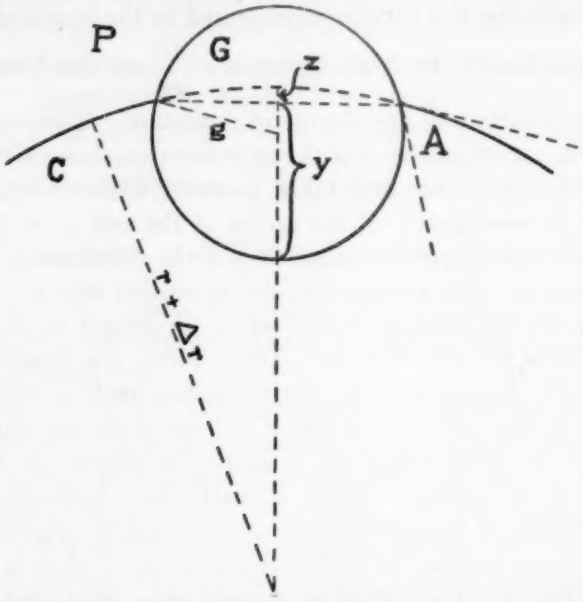

FIG. 3. Diagram showing geometrical construction used in calculating surface areas involved, as a solid spherical particle of radius g is ingested by a liquid sphere (shown in part) of radius $4g$ or r. A is angle of contact.

The error of Tait's statement and of the predictions based upon it is evident from the figure. The minimum surface energy is not

[7] By simple geometrical and trigonometrical consideration of Fig. 3 it is found that

$$\cos A = \frac{(r + \Delta r)^2 + g^2 - (r + \Delta r + g - z - y)^2}{2g(r + \Delta r)}$$

This equation was used in calculating cos A as given in Table I.

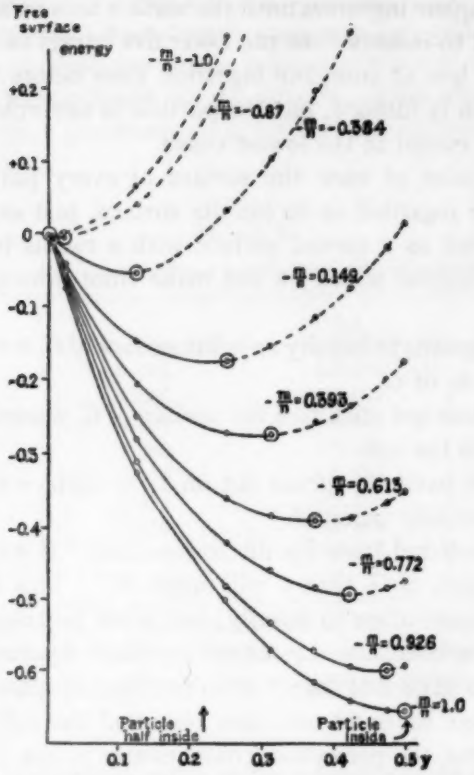

FIG. 4. Graphs showing how free surface energy (expressed in relative not absolute units) varies during the passage of a solid particle of radius 0.25, inside a hypothetical cell of radius 1.0, for various values of $\frac{m}{n}$ where n and m represent the gain in free surface energy caused by a given increase in the area of the cell plasma and cell glass interfaces respectively. Abscissæ, y, vary with the position of the particle with respect to the cell, half inside at $0.22y$ and completely inside at $0.5y$. Points marked by large circles are points of equilibrium where surface energy is at a minimum, and represent also positions of cell and particle where the values of m and n used in calculating that curve would be in equilibrium at the angle of contact. The curves are dotted beyond these points to indicate that work must be done upon the particle to complete its ingestion since the surface energy is increasing. Values of $-\frac{m}{n} = \cos A$ are given in Table I. See text for further discussion.

reached at complete ingestion until the surface tension is such that the cell will spread to infinity. In the lower five curves in Fig. 4 the surface energy is less at complete ingestion than before ingestion; *i.e.*, Tait's condition is fulfilled, but the particle is nevertheless only partially ingested except in the lowest curve.

From this point of view the surface of every particle, however small, must be regarded as an infinite surface, just as a flat surface may be regarded as a curved surface with a radius infinitely large. The only predictions which we can make from principles of surface tension are:

1. If a cell spreads to infinity on a flat surface of *G*, it will completely ingest a particle of *G*.

2. If a cell does not stick to a flat surface of *G*, a small particle of *G* will not stick to the cell.

3. If a cell is partially spread out on a flat surface of *G*, a particle of *G* will be partially ingested.

Tait has predicted from his discussion that: "If a substance, *C*, is unstable on a slab of *G*, then *C* will ingest *G*." This is true if "unstable" means spreading to infinity; but when he tries to prove this prediction experimentally by observing that hyaline thigmocytes are unstable on glass and ingest small particles of glass, it may fairly be objected that he could not have observed the cells spreading to infinity and that the prediction consequently is not fulfilled by the facts. The explanation of the experiment is either that the cell was too rigid to spread to infinity on a flat surface though it could do so readily on the curved surface of a small particle, or that the equilibrium observed on a flat surface was a true surface tension equilibrium and that the small force of surface tension which prevented the small particle from being completely ingested was easily overcome by the ameboid movement (changes in consistency) of the cell.

A similar explanation may be given when Tait predicts that: "If *C* ingests a particle of *G*, then *C* (may or) may not be unstable on a slab of *G*," and adds in consequence that "the fact that stable cells such as mammalian polymorphonuclears do not spread extensively on glass is no evidence that they ingest by other than physical means." The true prediction from principles of surface tension is that if *C* ingests *G*, it will spread to infinity on a slab of *G*, and the ob-

served behavior of leucocytes means either that the rigidity of the cell prevents its reaching a true equilibrium on a flat surface, or that it ingests, by its ameboid movement, a small particle of G which otherwise would be merely stuck on the outside. Both alternatives seem equally probable. Obviously such tests of the surface tension hypothesis are worthless.

TABLE I.

Areas of Contact Surfaces during Ingestion of a Particle.

y	s	Δx	$\cos A$
0.5	0.785	+0.131	1.00
0.475	0.746	+0.092	0.926
0.425	0.667	+0.024	0.772
0.375	0.588	−0.028	0.613
0.312	0.490	−0.082	0.393
0.250	0.393	−0.118	0.149
0.125	0.196	−0.130	−0.384
0.025	0.039	−0.037	−0.870
0	0	0	−1.0

y expresses the degree of ingestion as shown in Fig. 3. Ingestion is complete at $0.5y$, one-half complete at $0.22y$. s is the area of the cell in contact with the particle; Δx the change in the area of the cell plasma interface; A is the angle of contact.

Is it easier to ingest a small particle than a large particle, and if so, why? Tait says: "If in the case of a given phagocytic cell we have a certain volume of material which can just be ingested as a spherical piece, that cell will ingest the same volume more readily when the material is either sub-divided or distorted from the spherical form for by this means s is increased without change in x [Δx in our terminology]." It is true from principles of surface tension that a large particle will be less completely ingested than a small one for the same values of m and n, *i.e.*, for the same contact angle, but the same limiting values of m and n (*i.e.*, $-\frac{m}{n} = 1$) are necessary for complete ingestion of any particle of any shape or size. It is true, however, that a small particle could be more easily pulled inside against the force of surface tension from some equilibrium position in the surface than

a large particle which made the same angle of contact with the cell. Also, it is easier for a cell to spread around a small particle than around a large one because the necessary mechanical deformation is less.

Phagocytosis has been described repeatedly as taking place in two stages (Kite and Wherry (3)), the actual ingestion being preceded by a phase in which the object is merely stuck on the outside. The preliminary stage is clearly a surface tension phenomenon. The frequency of its occurrence is due to the fact that the surface of the cell exposed to the plasma is thereby decreased.[8] When objects seem to be permanently stuck on the outside of a cell this may be a true surface tension equilibrium or, more likely, it may be that the rigidity of the structure of the interior of the cell prevents the further deformation necessary to reach a true surface tension equilibrium with the object completely inside.

In conclusion emphasis may be laid upon the significance, from the point of view of surface tension of Tait's general proposition which states that only unstable cells tend to be phagocytic.

SUMMARY.

The theoretical behavior of a hypothetical fluid cell in contact with flat and curved solid surfaces is discussed from the point of view of surface tension.

An equation is derived for calculating the equilibrium position of the cell on a flat surface in terms of the surface tensions between the cell and the plasma, the plasma and the solid surface, and the solid surface and the cell. It is shown that the same equilibrium is predicted from consideration of the contact angle between the cell and the solid body.

The relative surface energy has been calculated at various stages in the ingestion of a solid particle by a fluid cell four times as large in diameter, and it is thus shown that no particle will be ingested until the surface tensions are such that the cell would spread to infinity on a flat surface of the same substance. Here again the same equilibrium is predicted from considerations of the contact angle.

[8] This decrease is evidenced in Table I by the negative values of Δx when y is 0.375 or less.

The adhesiveness of blood cells to solid substances is shown to be a pure surface tension phenomenon, but in most reactions between living cells and solid bodies the fluidity of the protoplasm is also a factor of prime importance.

The frequent occurrence of adhesiveness as a property of cells in contact with solid bodies is due in part to the fact that, by so adhering, the surface area of the cell not touching the solid is decreased.

Careful criticisms of this manuscript by Dr. W. T. Bovie and Dr. E. K. Carver are gratefully acknowledged.

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CHANGE OF ACID AGGLUTINATION OPTIMUM AS INDEX OF BACTERIAL MUTATION.

By PAUL H. De KRUIF.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, December 16, 1921.)

INTRODUCTION.

Two distinct varieties of microbe have been shown to exist in cultures of the bacillus of rabbit septicemia (1). These have been designated as Microbes D and G. Microbe D is the variety isolated from rabbits dead of spontaneous infection with the rabbit septicemia bacillus. It is characterized by diffuse growth in serum and plain broth, forms opaque fluorescing colonies on serum agar, and is highly virulent for rabbits. Microbe G, first discovered accidentally in Microbe D cultures, has been proved to be a true mutant of the parent D form (2). The mutant Microbe G grows in granular fashion in liquid media, forms translucent bluish colonies with no fluorescence, and exhibits extremely low virulence for rabbits. The mutation experiments demonstrating that Microbe D, under controllable conditions, changes into Type G were performed with D strains arising from single individuals isolated by Barber's pipette.

The granular growth of Microbe G in fluid medium is one of its most striking differential characters, and has persisted throughout transplants for more than 1 year. This sedimenting growth of Type G in broth, compared to the evenly suspended, uniformly turbid appearance of broth cultures of Type D led to an examination of the acid agglutination optima of the two types.

Methods.

The method for the determination of the acid agglutination optimum was that of Michaelis (3), later described in full by Beniasch (4). It consisted in mixing carefully prepared suspensions of the organism

to be tested with equal volumes of buffer mixtures of varying C_{H^+}. Two buffer series were employed, Na lactate-lactic acid and Na acetate-acetic acid. The mixtures were made according to Tables I and II.

TABLE I.

Na Lactate-Lactic Acid Series.

pH	4.7	4.5	4.1	3.8	3.5	3.3	3.0	2.7	2.4
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Na Lactate N/10.....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Lactic acid N/10	0.06	0.12	0.25	0.5	1.0	—	—	—	—
Lactic acid N	—	—	—	—	—	0.2	0.4	0.8	1.6
Distilled water.....	1.54	1.48	1.35	1.1	0.6	1.4	1.2	0.8	—

TABLE II.

Na Acetate-Acetic Acid Series.

pH	5.6	5.35	5.05	4.75	4.4	4.1	3.8	3.5	3.2	
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	
Na Acetate N/10.....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Acetic acid N/10	0.06	0.12	0.25	0.5	1.0	—	—	—	—	Final volume, 2.1 cc.
Acetic acid N	—	—	—	—	—	0.2	0.4	0.8	1.6	
Distilled water.....	1.54	1.48	1.35	1.1	0.6	1.4	1.2	0.8	—	

Preparation of Suspensions.

Microbe G sediments rapidly in fluid media. It fails to remain in even suspension when the sediment from a centrifuged culture is taken up in 0.85 per cent NaCl. If, however, such sediments are repeatedly washed in large volumes of distilled water, very stable suspensions can be obtained. In order to secure perfect comparability, Microbe D was treated in a similar manner, although in this case washing with distilled water is unnecessary and it yields stable suspensions in 0.85 per cent NaCl. The technique of preparation of suspensions was as follows.

5 per cent rabbit serum broth cultures, 24 hours incubation, were centrifuged, the supernatant fluids discarded, and the sediments thoroughly shaken in a volume of distilled water equal to that of the

original culture. The centrifugation and resuspension in distilled water were repeated four times in all. The final suspensions were carefully brought to a uniform turbidity.

EXPERIMENTAL.

All experiments were carried out by adding 1 cc. of distilled water suspension of the microbe in question to an equal volume of each of the buffer mixtures just described. The tubes were carefully shaken, placed in the water bath at 43°C. and readings taken at 1, 2, and 16 hours.

TABLE III.

Acid Agglutination Optimun of Microbes D and G, Strain R 15. Na Lactate-Lactic Acid Buffer Series.

Organism.	Tube No.	1	2	3	4	5	6	7	8	9	Optimum pH. Final reading.
	pH	4.7	4.5	4.1	3.8	3.5	3.3	3.0	2.7	2.4	
Microbe D.	1 hour.	0	0	0	0	+	+	0	0	0	3.5–3.3
	2 hours.	0	0	0	0	++	++	+	+	Tr.	
	16 "	0	0	0	0	C	C	+	Tr.	Tr.	
Microbe G.	1 hour.	Tr.+	+	+	++	+	0	0	0	0	4.7–3.8
	2 hours.	Tr.+	++	++	C	++	0	0	0	0	
	16 "	C	C	C	C	+	Tr.+	0	0	0	

In this and the following tables lesser degrees of floculation are recorded as ++, +, and Tr. (trace). C indicates complete flocculation.

The agglutination optimum was considered to be that zone of C_{H^+} where complete flocculation occurred; that is, where the microbes sedimented so perfectly as to leave a water-clear supernatant fluid. Lesser degrees of agglutination are recorded as ++, +, and Tr. (trace). The readings are greatly facilitated by holding the tubes before a powerful beam of light, projected downward in front of a dark background.

Experiment 1. Acid Agglutination Optimum, Microbes D and G, Strain R 15, in Na Lactate-Lactic Acid Buffer Series.—Suspensions of Microbes D and G, isolated from Strain R 15, bacillus of rabbit septicemia, were tested against the Na lactate-lactic acid buffer series. The experiments were carried out as described above. The results are recorded in Table III.

The results recorded in Table III show a definite difference in acid agglutination optimum of the two varieties. The readings, taken at 1,2, and 16 hours, indicate that the reaction does not take place with the speed of that of immune agglutination of the majority of bacteria. It is necessary to allow ample time to elapse before taking the final readings. 16 hours have been found to be sufficient, no material change in readings being noted after this time.

The same suspensions were tested against the Na acetate-acetic acid buffer series, with a similar result recorded in Table IV. Temperature, as before, was 43°C. in the water bath.

TABLE IV.

Acid Agglutination Optimum of Microbes D and G.
Na Acetate-Acetic Acid Series.

Organism.	Tube No.	1	2	3	4	5	6	7	8	9	Optimum pH.
	pH	5.5	5.35	5.05	4.75	4.4	4.1	3.8	3.5	3.2	
Microbe D.	1 hour.	0	0	0	0	0	0	0	+	+	3.5 –3.2
	2 hours.	0	0	0	0	0	0	0	C	C	
	16 "	0	0	0	0	0	0	0	C	C	
Microbe G.	1 hour.	0	0	0	+	++	++	+	0	0	4.75–3.8
	2 hours.	0	0	0	++	C	C	++	0	0	
	16 "	Tr.+	Tr.	++	C	C	C	C	+	Tr.	

The results in the case of the Na acetate-acetic acid series correspond to those of the Na lactate-lactic acid mixtures. The final readings are identical, the only difference lying in a slightly more rapid flocculation in the acetate series.

Variability of Optimum of Microbe G as Compared to that of Microbe D.

A number of strains were now collected, the D and G types isolated and subjected to test with the Na acetate-acetic acid series. The result (Table V) confirms the findings of the previous experiments. Final reading was made after 16 hours with incubation at 43°C. The results are presented in Table V.

It will be noted that the acid agglutination optimum for Microbe D is the same in case of all three of the strains tested. On the other hand, the optimum for Microbe G varies to a considerable extent. This variation is never so great as to prejudice its value as a criterion of differentiation from the parent D form. In all cases complete flocculation of Type G occurs at a distinctly lower $C_H{}^+$ than that of Type D. The difference between the two types in regard to the smallest amount of hydrogen ion in which complete flocculation takes place is never less than 0.6 pH. In short, the organism in the process of mutation gains in sensitivity to flocculation in the presence of H ions.

TABLE V.

Acid Agglutination Optima of D and their Mutant G Forms. Na Acetate-Acetic Acid Series.

Strain.	Tube No.	1	2	3	4	5	6	7	8	9	Optimum pH.
	pH	5.6	5.35	5.05	4.75	4.4	4.1	3.8	3.5	3.2	
R 15	D-S 49	0	0	0	0	0	0	0	C	C	3.5 –3.2
	G-S 52	Tr.	Tr.	++	C	C	C	C	+	Tr.	4.75–3.8
	G-S 28*	Tr.	Tr.	C	C	C	C	C	++	++	5.05–3.8
R 11	D-S 43	0	0	0	0	0	0	0	C	C	3.5 –3.2
	G-S 42	0	0	+	+	+	C	C	C	+	4.1 –3.5
R 22	D-S 31	0	0	0	0	0	0	Tr.	C	C	3.5 –3.2
	G-S 32	0	Tr.	+	+	C	C	C	++	Tr.	4.4 –3.8

* G-S 28, a mutant from the same parent D strain as G-S 52.

Sobernheim and Seligmann (5) found a strain of *Bacillus enteritidis* to separate into two races. Beniasch (4) tested the acid agglutination point of this organism and found it to have altered its acid agglutination optimum when tested on two different occasions, a year having elapsed between the two tests. In this work apparently no attempt was made to establish the occurrence of a mutation, or to separate the two varieties.

Variations in the Agglutination Optimum of Type G.

Table V indicates that the agglutination optima of various strains of Microbe G are not as strictly uniform as those of the parent D type. One of the causes of this variation is passage of the microbe through the animal body. An example of this variation was observed during an attempt to cause reversion of Microbe G to the parent D form.

The Type G strain in question was characteristically of very low virulence. 1.0 cc. of a serum broth culture, injected intrapleurally, was required to produce fatal infection of a 600 gm. rabbit. The organism recovered at necropsy of this animal was cultured and injected into a second animal, and so on. At the third animal passage

TABLE VI.

Effect of Animal Passage on Acid Agglutinability of Type G.

	pH	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5	3.2
Microbe R 15 G	Before passage.	Tr.	Tr.	++	C	C	C	C	+	Tr.
	After three animal passages.	C	C	C	C	C	C	C	C	C

the virulence had greatly increased, 10^{-4} cc. of a serum broth culture being fatal. But the organism, far from returning to the uniformly turbid growth character of the Type D form, became more intensely granular in its growth. This characteristic was so marked that difficulty was experienced in preparing the washed suspensions for acid agglutination test.

The acid agglutination reaction of Type G strain after animal passage was compared with the same strain which had been transplanted in parallel in serum broth. The Na acetate-acetic acid buffer series was used. The culture was incubated at 43°C. for 16 hours (Table VI).

It will be seen from Table VI, first, that much less hydrogen ion is required to produce complete flocculation, and second that the optimum is very greatly broadened. It has been widened from

pH 4.7 to 3.8 before animal passage, to pH 5.6 to 3.2 after passage through three rabbits.

Up to the present, the change in acid agglutination optimum that occurs during mutation has been accompanied invariably by a great loss in virulence. For example, all Type D strains tested have been fatal to rabbits in doses of 10^{-5} to 10^{-7} of a serum broth culture. The Type G forms arising from such strains are seldom fatal in 0.5 cc. of undiluted culture. Frequently rabbits are able to resist 1.0 cc.

The experiment just described indicates that the decrease in stability to acid does not necessarily go hand in hand with loss of virulence, and certainly bears no causal relationship to such loss. For, while the stability to the hydrogen ion had greatly *decreased* during animal passage, the virulence had *increased* from 0.5 cc. to 10^{-4} cc.

SUMMARY AND CONCLUSIONS.

A distinct difference in acid agglutination optimum for Type D (bacillus of rabbit septicemia) and its mutant form, Type G, has been observed. The optimum for Type D lies between pH 3.5 and pH 3.0. This changes during mutation, the resulting Type G mutants having in general an optimum lying between pH 4.7 and pH 3.8.

The constancy of the optimum for Type D is very strict, while that for Type G is slightly less so. The variation is never so great as to cause an overlapping of optima and consequent failure of differentiation.

These acid agglutination optima are in the nature of physical constants for the two types and would imply a fundamental difference in the chemical constitution of the organisms.

Animal passage, far from causing a reversion of the mutant Type G to the primordial Type D form, brings about a still greater instability in the presence of H ions.

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THE MECHANISM OF GRANULAR GROWTH OF RABBIT SEPTICEMIA BACILLUS TYPE G.

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INTRODUCTION.

It has been stated by Beniasch (1) that the acid flocculation optimum of bacteria is referable only to the $C_H{}^+$ and is not influenced by the unionized acid or the anion of the acid. This concept may hold for buffer mixtures made up of certain acids and their sodium salts. But certain buffer mixtures exist which are composed of an acid plus some totally different substance, bearing no close chemical relationship to the acid. Examples of such buffer mixtures are glycocoll-HCl and glycocoll-Na acetate-NaH_2PO_4-HCl. These have been found by the writer (2) to be very useful in acid agglutination studies. It is important to learn whether such buffer substances exert an effect on the acid agglutination point of bacteria, which might differ from the values found for the Michaelis (3) series.

EXPERIMENTAL.

The Na lactate-lactic acid buffer series of Michaelis (3) covers a range from pH 4.7 to 2.4. It was desired to test the behavior of distilled water suspensions of Microbe G and D in higher $C_H{}^+$. Since this range was not covered by the Na lactate-lactic acid series, recourse was had to the glycocoll-HCl series of Sörensen (4), which covers a range from pH 3.0 to pH 1.2. This buffer series was prepared from the Sörensen chart.

The pH of these mixtures was tested colorimetrically and checked by the potentiometer.

The flocculating activity of this buffer series was then compared to that of the Na lactate-lactic acid series. The technique of the

experiments was identical with that described in the preceding paper (5). 1 cc. of the buffer mixtures was added to equal volumes of four times washed distilled water suspensions of Microbes D and G, bacillus of rabbit septicemia. The mixtures were placed in the water bath at 43°C. for 16 hours, and readings taken. The results of this experiment are recorded in Table I.

TABLE I.

Agglutination of Microbes D and G in Na Lactate-Lactic Acid and Glycocoll-HCl Buffer Series.

	pH	4.7	4.5	4.1	3.8	3.5	3.3	3.0	2.7	2.4		Complete agglutination.
												pH
Na lactate-lactic acid.	Microbe D.	0	0	0	++	C	C	+	+	0		3.5–3.3
	Microbe G.	C	C	C	++	+	0	0	0	0		None at 2.7–2.4. 4.7–4.1
												None at 3.3–2.4.
	pH	3.0	2.8	2.6	2.4	2.2	2.0	1.8	1.6	1.4	1.2	
Glycocoll-HCl.	Microbe D.	C	C	C	C	++	++	++	++	+	0	3.0–2.8
	Microbe G.	++	++	+	+	+	0	0	0	0	0	++ 3.0–2.8

In this and the following tables the mixtures were kept in the water bath for 16 hours at 43°C. C indicates complete flocculation; Tr., trace.

The results given in Table I indicate that other factors besides the C_H+ are important in the interpretation of the acid agglutination point of the organism in question. In the case of Microbe D, Na lactate-lactic acid series, for example, complete flocculation occurs at pH 3.5 to 3.3, slight at pH 3.0, a trace at pH 2.7, and none at all at pH 2.4.

On the other hand, Microbe D in the glycocoll-HCl series, flocculates completely at pH 3.0, 2.8, 2.6, and 2.4. This difference of effect in the two buffer series is illustrated graphically in Fig. 1.

A similar result occurs in the case of Microbe G which flocculates completely at pH 4.7, 4.5, and 4.1, and not at all at pH 3.0 to 2.4, in the Na lactate-lactic acid series. Yet ++ agglutination occurs at

pH 3.0 and 2.8 and + at pH 2.6 to 2.2 of the glycocoll-HCl series. It would appear that the glycocoll or the anion of the acid has the property of broadening the acid agglutination optima of Microbes D and G, or of shifting the optima toward a zone of higher C_H+.

These results led to experiments which suggest an explanation for the granular growth of Microbe G in plain broth. Washed suspensions of this organism in distilled water, pH 6.0, do not sediment. On the other hand, rapid sedimentation occurs in plain broth at pH 7.4 to 7.0. This leads to the conclusion that broth contains a constituent which, *per se*, agglutinates the Type G organisms, or which has the property, like glycocoll, of shifting their acid agglutination optimum.

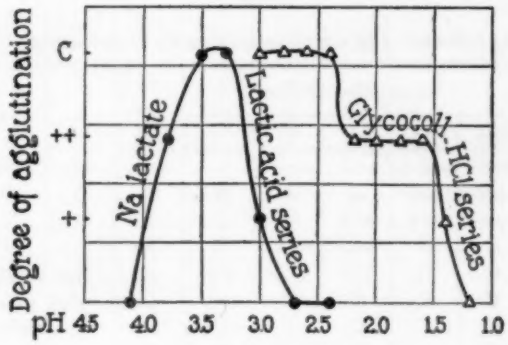

FIG. 1.

Preliminary experiments were made in which the flocculating effect of the various components of plain broth were tested against washed distilled water suspensions of Microbe G. The constituents tested were beef infusion, peptone, and Na_2HPO_4.

Beef Infusion.—500 gm. of chopped beef were extracted in 1,000 cc. of tap water, in the ice box for 16 hours. The mixture was then boiled for 30 minutes, filtered, titrated to pH 7.4, refiltered, and sterilized in the autoclave.

Peptone Solution.—10 gm. of Fairchild's peptone were dissolved in 1,000 cc. of distilled water, boiled for 30 minutes, filtered, adjusted to pH 7.4, and sterilized in the autoclave.

Na_2HPO_4.—10 gm. of this salt were dissolved in one liter of distilled water adjusted to pH 7.4, and sterilized in the autoclave.

Of these three solutions, at pH 7.4, beef infusion alone showed marked flocculating activity against Microbe G.

The flocculating effect of beef infusion at varying acidities was now tested. The beef infusion solution just described was titrated with N/10 HCl to increasing degrees of acidity, from pH 7.5 to pH 2.0. The beef infusion at all of these C_H+ was now diluted with distilled H_2O, the dilutions increasing from 1:2 to 1:40. Each of these dilutions at each pH, was now added in equal volume to 1 cc. of distilled water suspensions of four times washed Microbes D and G. The mixtures were kept in the water bath for 16 hours at 43°C., and readings taken. The results are recorded in Tables II and III.

TABLE II.

Beef Infusion Agglutination of Microbe G at Varying C_H+.

pH	Amount of beef infusion.							Result.
	1.0 per cent	0.75 per cent	0.5 per cent	0.25 per cent	0.2 per cent	0.1 per cent	0.075 per cent	
7.5	++	++	++	+	+	0	0	
7.0	C	++	++	++	+	+	0	
6.5	−	C	C	++	++	+	+	
6.0	−	C	C	++	++	++	+	Final volume of beef infusion dilution = 1.0 cc.
5.0	−	C	C	C	C	++	+	+ distilled H_2O suspension G 1.0 cc.
4.5	−	C	C	C	C	C	C	
4.0	−	C	C	C	C	C	C	
3.5	−	C	C	C	++	++	+	
3.0	−	C	C	C	++	++	+	
2.5	−	C	C	++	++	+	+	
2.0	−	C	C	++	+	+	+	

Table II shows for Type G, that, as the acidity increases down to to pH 4.5, the amount of beef infusion necessary to cause complete agglutination becomes less and less. At pH 4.5 to pH 4.0 traces of beef infusion cause complete agglutination. This point corresponds to the acid agglutination optimum of Microbe G in various buffer series. Beyond this point, that is, at pH $<$4.0, increasing amounts of beef infusion are again necessary to produce complete flocculation.

Table III shows the same effect in the case of Microbe D, the only difference being that complete flocculation of the D type by a given

concentration of beef infusion demands a higher C_H+ than in the case of Type G. For each organism the range of C_H+ at which the smallest amount of beef infusion is required for complete sedimentation is precisely the optimum zone of acid agglutination described in the preceding paper.

The results of the experiments recorded in Tables II and III are represented graphically in Fig, 2, in which the pH of the various dilutions of beef infusion are plotted on the abscissæ against the *recip-*

TABLE III.

Beef Infusion Agglutination of Microbe D at Varying C_H+.

pH	Amount of beef infusion.							Result.
	1.0 per cent	0.75 per cent	0.5 per cent	0.25 per cent	0.2 per cent	0.1 per cent	0.075 per cent	
7.5	Tr.	0	0	0	0	0	0	
7.0	+	+	+	Tr.	0	0	0	
6.5	−	C	C	++	+	0	0	Final volume of beef infusion dilution = 1.0 cc. + distilled H_2O suspension D 1.0 cc.
6.0	−	C	C	++	+	Tr.	Tr.	
5.0	−	C	C	C	++	+	+	
4.5	−	C	C	C	C	++	++	
4.0	−	C	C	C	C	C	C	
3.5	−	C	C	C	C	C	C	
3.0	−	C	C	C	++	++	++	
2.5	−	C	C	C	++	++	++	
2.0	−	C	C	++	++	+	+	

rocals of the amount of beef infusion on the ordinates. That is, 1.0 cc. of beef infusion is represented by 1, 0.5 cc. by 2, 0.5 cc. by 4, and so on. The points recorded on the graphs are in all cases the amounts of beef infusion which cause *complete flocculation*, represented by C, Tables II and III.

The graphs of Fig. 2 would seem to afford an explanation for the granular growth of Microbe G and the diffuse growth of Microbe D in broth, since at pH 7.0, the C_H+ at which these organisms are grown, large amounts of beef infusion cause complete sedimentation of Microbe G and little or no agglutination of Microbe D.

What is more, the graphs would appear to indicate that beef infusion, *per se*, does not cause the agglutination. It merely widens the

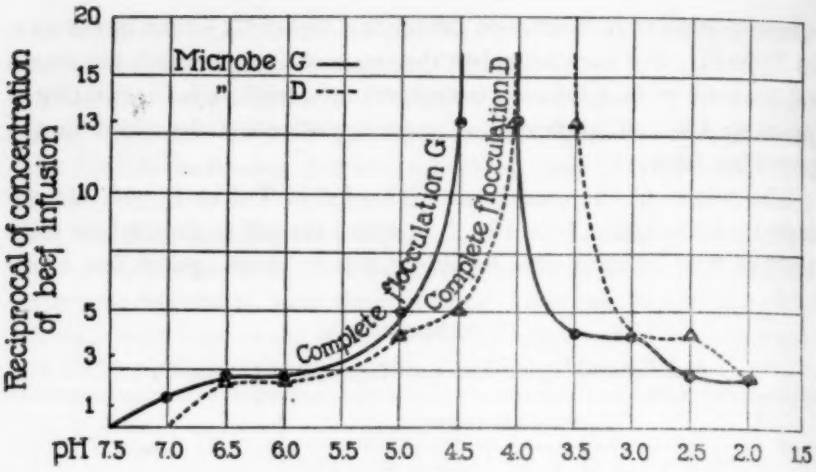

FIG. 2.

acid agglutination optimum zone. This is clear from the fact that small amounts of beef infusion do not cause a flocculation independent of the C_H+.

Suspensions of Types D and G were similarly tested against decreasing concentrations of peptone (Fairchild) at various C_H+. The technique of the experiments was identical with that for beef infusion. The peptone solution from which the various dilutions were made

TABLE IV.

Peptone Agglutination of Microbe G at Varying C_H+.

pH	Amount of peptone.							Result.
	1.0 per cent	0.5 per cent	0.25 per cent	0.2 per cent	0.1 per cent	0.075 per cent	0.05 per cent	
7.5–6.0	0	0	0	0	0	0	0	
5.0	C	++	Tr.	0	0	0	0	
4.5	C	C	Tr.	0	0	0	0	Final volume of peptone dilutions 1.0 cc. + distilled H_2O suspension G 1.0 cc.
4.0	C	C	C	C	++	Tr.	0	
3.5	C	C	C	C	C	C	+	
3.0	C	C	C	C	C	C	C	
2.5	C	C	C	C	C	C	C	
2.0	C	+	+	Tr.	0	0	0	

was of 1 per cent concentration in distilled water. The results are recorded in Tables IV and V.

TABLE V.

Peptone Agglutination of Microbe D at Varying C_H+.

pH	Amount of peptone.							Result.
	1.0 per cent	0.5 per cent	0.25 per cent	0.2 per cent	0.1 per cent	0.075 per cent	0.05 per cent	
7.5–3.5	0	0	0	0	0	0	0	Final volume of peptone dilutions 1.0 cc. + distilled H_2O suspension D 1.0 cc.
3.0	0	0	C	C	C	C	Tr.	
2.5	0	C	C	C	C	C	C	
2.0	0	0	C	C	++	++	++	

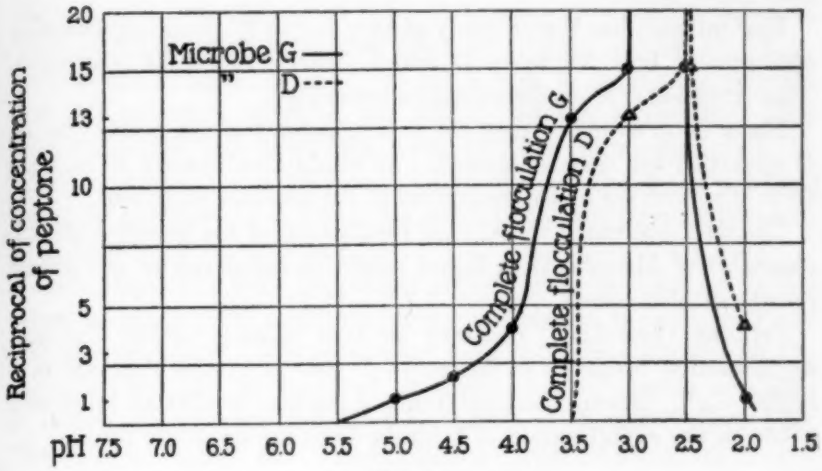

FIG. 3.

It will be observed from Tables IV and V that the results are strikingly different from those obtained with beef infusion. Peptone, contrary to beef infusion, appears to shift the optimum zone of agglutination in the direction of a higher C_H+, and effect analogous to that observed in the glycocoll-HCl buffer mixtures (Table I).

The optimum for Type G in the lactate and acetate buffer mixtures lies between pH 5.0 and pH 3.8. Peptone changes the optimum to pH 3.0 to 2.5. For Type D, an analogous effect is observed, the

optimum shifting from pH 3.5 to 2.5. The results recorded in Tables IV and V are graphically represented in Fig. 3.

It is interesting to observe that for Microbe D strong concentrations of peptone (0.5 and 0.25 cc. of 1 per cent solution) actually suppress flocculation completely at pH 3.0. The effect of peptone, contrary to that of beef infusion, would appear to be a stabilizing one.

SUMMARY.

The acid agglutination optimum of Microbes D and G is not independent of the nature of the buffer mixture. Glycocoll-HCl buffer mixtures cause complete flocculation at high C_H+ (2.7 to 2.4), at which points little or no flocculation occurs with the Na lactate-lactic acid buffer series.

Beef infusion has the property of broadening the acid agglutination optimum of both Microbes D and G, bacilli of rabbit septicemia. This extension is in the direction of a lower C_H+.

There is no evidence that the beef infusion has the power, *per se*, of agglutinating these organisms. It would seem merely to increase their sensitiveness to sedimentation in the presence of H ions.

The data presented explain the mechanism of the granular growth character of Microbe G in liquid media as compared to the diffuse growth of Microbe D.

Peptone (Fairchild), contrary to beef infusion, shifts the acid agglutination optimum of Microbes D and G in the direction of a higher C_H+. Strong concentrations of peptone exhibit an inhibitory effect on the agglutination of Microbe D in the optimum zone.

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THE AGGLUTINATION OF RED BLOOD CELLS IN THE PRESENCE OF BLOOD SERA.

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The optimum for the agglutination of normal sheep cells in isotonic saccharose solution has been given as pH 4.75.[1] To correct a possible error in the colorimetric measurements originally employed electrometric determinations have been made in a similar series of experiments in which graduated amounts of N/10 to N/40 HCl have been added to suspensions of red blood cells in saccharose solution and measurements made of the reaction of the supernatant fluid from which the cells have been removed 15 to 30 minutes after the addition of acid. The average pH 4.76 of the following values thus found corresponds closely with the result of the colorimetric method: pH 4.55, 4.57, 4.79, 4.90, and 5.03.

Cells sensitized with approximately 10 units of immune rabbit serum at pH 5.3, the optimum for combination of the cells with the immune sensitizer[2] and washed with pure saccharose solution at the same reaction agglutinate most promptly at the following reactions in a series of experiments: pH 5.22 to 5.45, 5.26, and 5.06 to 5.30. The average is pH 5.26. The colorimetric method had given from a larger series the value pH 5.3. If the cells be not washed after the addition of immune serum which was present in a concentration of 0.5 per cent by volume, the optimum occurs at a slightly higher figure, pH 5.5 approximately.

If a similar small volume of active normal rabbit serum be added to the cells in place of the immune serum, the optimum for agglutination occurs at the same point, pH 5.5.

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[1] Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 309.

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The euglobulin precipitates most promptly and completely from rabbit serum diluted 1:20 with distilled water at the same reaction, pH 5.5, and it is apparent that the agglutination of the cells is intimately related to the precipitation of the serum euglobulin.

The same relation is observed in the agglutination of sheep cells to which a like small amount of their own active serum has been added, as shown by the following values for the optimal point for agglutination in the presence of homologous serum: pH 5.58, 5.44, and 5.38. The euglobulin itself precipitates best from sheep serum diluted 1:20 with distilled water at approximately pH 5.5.

Guggenheimer[3] has made an observation which corresponds closely with this, that if defibrinated sheep blood be washed directly with isotonic saccharose solution the euglobulin of the serum is carried down with the cells and will serve as the mid-piece fraction of complement to persensitize the cells on the subsequent addition of sensitizer.

The relation mentioned is noted again when sensitized sheep cells in saccharose solution are persensitized by the addition of active normal guinea pig serum. If such serum be added in the amount of 8 per cent of the total volume to an emulsion of sensitized cells of such concentration that one unit of complement is present, the optimal point for agglutination has been found at the following electrometric values: pH 6.19, 6.35, and 6.15. Five other experiments in which the estimation was made colorimetrically gave values between pH 5.9 and 6.3. The euglobulin has been found to precipitate best from guinea pig serum diluted 1:20 with distilled water between pH 6.2 and 6.4 (electrometric).[4]

If the cells were persensitized at pH 6.2 and washed by allowing them to settle spontaneously from pure saccharose solution of pH 6.0 the optimal point of agglutination was noted at the following reactions (electrometric): pH 5.71, 5.79, 5.76 to 6.18, 5.38 to 5.80, 5.78, and 5.69 to 5.77. This shift toward a more acid zone runs parallel with that observed in the precipitation of guinea pig globulin which has been washed as precipitate and redissolved by bringing to pH 7.4 with NaOH. Precipitation then has its optimum between pH 5.1 and 5.7.[4]

[3] Guggenheimer, H., *Z. Immunitätsforsch. Orig.*, viii, 1910–11, 295.
[4] Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 771.

A specific reaction in the immunological sense may be supposed to take place between the sheep cells and the immune or native sensitizers of rabbit or guinea pig serum. A reaction of the same nature can hardly be thought of as occurring between sheep cells and their own serum, and yet these sera act alike as protective colloids to sheep cells since in their presence agglutination of the cells is not observed at pH 4.7; and further, they sensitize the cells to agglutination at the characteristic reactions of the serum euglobulins.

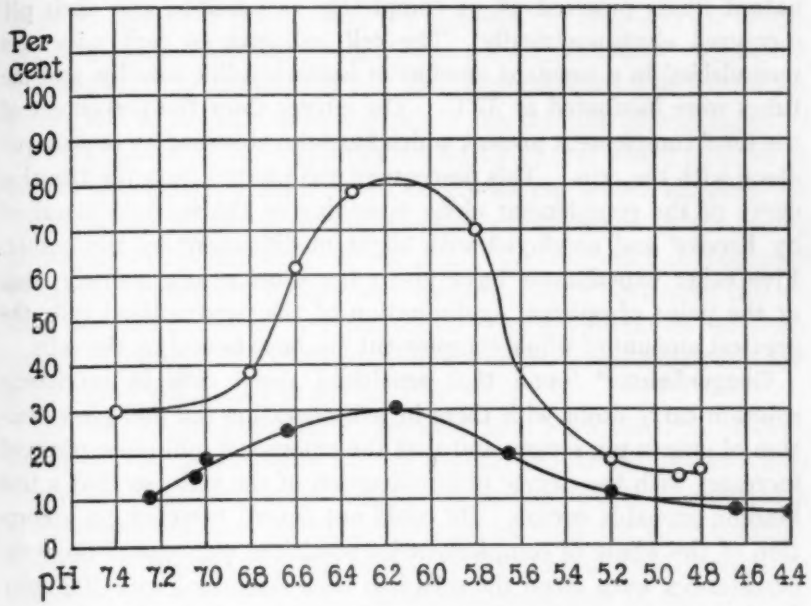

FIG. 1. Each curve represents a single experiment and gives as ordinates the percentage of the total amount of complement added which has been absorbed by sensitized cells at 4°C., with varying H ion concentrations as abscissæ.

If the cells from the tubes to which guinea pig serum has been added be sedimented and reemulsified in isotonic saline solution, it is found that they are not only persensitized, but within a certain range of reaction have combined apparently with the end-piece fraction of complement as well, since hemolysis occurs without further addition of end-piece. This is shown in the experiments recorded in the curves (Fig. 1). The phenomenon is observed regardless of whether the

cells have first been sensitized with rabbit serum or not; in the latter case the normal sensitizer of guinea pig serum, too small in amount to be recognized by the usual means, is probably united with the cells. In the experiments recorded cells were sensitized with approximately 8 units of sensitizer and added to tubes along with guinea pig serum in such amounts as to give a concentration of one unit of complement. N/10 NaOH and HCl were immediately added and the tubes kept at 4°C. for 45 minutes. The cells were then sedimented, the supernatant fluids pipetted off as completely as possible, and their pH measured electrometrically. The cell sediment in each tube was reemulsified in a constant amount of isotonic saline solution and the tubes were incubated at 37°C. The curves show the percentage of the total complement present which has been absorbed by or removed along with the cells. This percentage was plotted from the titration curve of the complement alone according to the method described by Brooks[5] and employed with slight modification[4] by the author. Five other experiments have given the same result; namely, that at the point of optimal agglutination of the persensitized cells the greatest amount of whole complement has been bound by the cells.

Guggenheimer[3] found that sensitized sheep cells in saccharose solution carry down with them in sedimentation the mid-piece fraction of guinea pig serum, and that the amount of mid-piece removed increases with the degree of sensitization of the cells, so that a true binding probably occurs. He could not detect, however, an absorption of the whole of complement by sensitized cells under these circumstances, even when the cells had been sensitized with 100 units. In the experiments described above in which such an absorption appears to have taken place a small amount of end-piece must have been present in the liquid phase of the sedimented cell mass, which could not have been removed by washing without disturbing the equilibrium relations between the cells and the sensitizer[2] and complement. According to the experience of Zinsser,[6] the trace of end-piece retained by the globulin sediment in the partition of complement by dialysis is sufficient to give complementary power to the globulin

[5] Brooks, S. C., *J. Med. Research*, 1919–20, xli, 399.

[6] Zinnser, H., Infection and resistance, New York, 1914, 180.

sediment alone. However, in the experiment recorded in the upper curve of Fig. 1, an hemolysis corresponding to an activity of 78 per cent of the total amount of complement originally present points distinctly to a true binding of end-piece by the persensitized cells.

It is generally believed that the protective and sensitizing effects of one colloid upon another with reference to precipitation by a third substance are due to a combination of some sort between the two colloids. There is no evidence available on which to base a judgement as to the nature of the combination between the cells and the serum euglobulins considered here, whether physical or chemical. However, the fact that the presence of the sera displaces the optimum for agglutination of the cells quite sharply to the reaction characteristic of the flocculation of the euglobulin added, or of that added last and in largest amount as in the case of the guinea pig serum, suggests the occurrence of a surface combination or condensation of the serum protein upon the surface of the red cell. The conclusion is supported by the observation of Porges[7] that bacteria which have been treated with such an excess of immune serum that agglutination does not appear, show the salt precipitation limits of the serum euglobulin and not those of the native bacteria.

The cells in the experiments reported here were not permeable to hemoglobin since hemolysis did not appear, and as will be mentioned later were very little permeable to inorganic ions, so that a penetration of the euglobulin into the interior of the cell does not seem possible. It would be difficult to explain furthermore how the small amount of serum protein relative to the mass of cells could give its own point of optimum flocculation to a mixture of cells and serum in any other way than as a surface deposition.

The euglobulin appears to be the active protein of the serum in combining with the cells.

A phenomenon based, as far as one may judge, upon precisely the same mechanism in the combination of a protein with a surface has been observed by Loeb.[8] This author has found that collodion membranes always acquire the characteristics of the protein with which

[7] Porges, O., *Centr. Bakt., Orig.* 1906, xl, 133.
[8] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 577.

they have been brought into contact, and that if such a membrane be treated with a solution of gelatin or oxyhemoglobin, for example, after the surplus protein has been washed away, the isoelectric point of the membrane is now that of the protein with which it has been treated.

This observation in connection with those reported here indicates the importance of factors which are non-specific in the serological sense in the mechanism of agglutination.

This view of the importance of the surface conditions in agglutination receives further support if the H ion concentration of the interior of the red cells be examined in relation to that of the fluid in which they are suspended This can be done by sedimenting the cells, removing the supernatant fluid and dissolving the cell sediment in a small amount of distilled water. Experiments which are shortly to be reported in full have shown that on the acid side of pH 7.4 at least, the reaction of the interior of fresh cells in a medium of low electrolyte content is maintained at a more alkaline level than that of the fluid outside. The following figures give the reactions of the outside fluid and of the dissolved cells in an experiment in which agglutination occurred at the two most acid reactions, although not promptly:

pH outside.	pH inside.
5.92	7.28
5.83	7.26
5.61	7.18
5.44	7.03
4.08	6.97

In numerous other experiments the suspending fluid has been brought very near pH 4.8 with the appearance of immediate agglutination, and the reaction of the dissolved cells found to lie between pH 7.20 and 6.8 if the reaction be measured within 15 minutes after the addition of acid.

The ionic state of the hemoglobin must be a large factor in the electric charge carried by the cell as a whole; the value pH 4.6, in the suspending fluid, as the isoelectric point of red blood cells[1] appears thus to correspond under the conditions of the determination with a

reaction within the cell of pH 6.8, which is the isoelectric point of hemoglobin.[9]

With older cells the reaction within the cell may be at pH 6.8 when that of the outside fluid is at the same point without the appearance of a trace of agglutination. The phenomenon of agglutination appears then to be related closely to an optimal reaction in the suspending fluid and probably of the cell membrane and not to a definite reaction in the interior of the cell.

CONCLUSIONS.

1. The addition of blood serum displaces the optimum for agglutination of red blood cells in a salt-free medium to the reaction characteristic of flocculation of the serum euglobulin.

2. This effect is not due merely to a mechanical entanglement of the cells by the precipitating euglobulin, since at reactions at which the latter is soluble it protects the cells from the agglutination which occurs in its absence.

3. A combination of some sort appears therefore to take place between sheep cells and sheep, rabbit, and guinea pig serum euglobulin, and involves a condensation of the serum protein upon the surface of the red cell.

4. At the optimal point for agglutination of persensitized cells both mid- and end-piece of complement combine with the cells.

5. Agglutination is closely related to an optimal H ion concentration in the suspending fluid, and probably of the cell membrane, and not to a definite reaction in the interior of the cell.

[9] Michaelis, L., and Takahashi, O., *Biochem. Z.*, 1910, xxix, 439. Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xli, 102. Michaelis, L., and Airila, Y., *Biochem. Z.*, 1921, cxviii, 144.

THE RELATIVE TOXICITY OF THE HALIDES AND CERTAIN OTHER ANIONS.

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When iodides are administered in ordinary doses excretion takes place rapidly. Heavy doses can usually be administered before toxic symptoms develop. The same is true to an even greater extent of bromides. The bromide ion appears to be capable of replacing the chloride ion to a considerable degree in the body fluid of mammals without the production of distinct toxic symptoms. Thus Frey (1910–11) has shown that while administration of sodium bromide (to rabbits) leads at first to increased excretion of sodium chloride, if the relative amounts in the diet are kept constant bromide and chloride excretion become parallel; while an animal kept for some time on a deficient chloride diet, and then fed bromide, tends to retain bromide equally with chloride.

There is evidence that some at any rate of the toxic symptoms following heavy administration of bromide are actually due to too great a depletion of chloride from the body, and not to the bromide ion (*cf.* Wolff and Opp, 1912).

After bromide administration hydrobromic acid occurs in the gastric juice (Wolff and Opp).

Similarly Herzfeld and Heimann (1911) have shown that after administration of iodide the excretion of chlorides and iodides is in inverse ratio. Hydriodic acid occurs in the gastric juice after iodide administration, and iodide is found in saliva, tears, perspiration, milk, sebum, and the secretion of the nasal mucous membrane (Cushny, 1918).

It is to be noted that Hale and Fishman (1908) state that bromide is excreted more slowly than corresponding doses of iodide.

According to Biedermann (1921), animal and vegetable diastases consist of a thermostable, organic component, by itself inactive, and a thermostable coenzyme. Numerous inorganic salts can act as the coenzyme, mainly through the action of their anions. The sodium salts in order of greatest effect are the chloride, bromide, nitrate, iodide, and sulphate.

The bromide ion distributes itself between blood corpuscles and plasma in a ratio (1:3.1) similar to that for the corresponding distribution of chloride ions (1 :2.1) (Wiechmann, 1921).

It is evident that bromides and iodides can to a certain extent replace chloride in the circulating and tissue fluids and secretions of the body with apparently the same functional effect, and without producing definite toxic symptoms. This research, carried out during the winters of 1919–20 and 1920–21, is directed toward the solution of the problem of to what extent such non-toxic replacement can take place.

In view of the fact that the iodine of sea water is present chiefly as iodate (Sonstadt, 1872, and Winkler, 1916) a comparative study of iodate has been included.

As a basis of comparison we have measured the survival times of frog heart and muscle-nerve preparations immersed in modified Locke solutions containing mixtures of the ions studied.

While much work has been carried out in determining the relative effects of various series of cations on the skeletal and cardiac muscles of the frog, less has been done with series of anions.

Loeb (1909) has shown that the relative effectiveness of halide ions in producing rhythmic contractions (a toxic effect) in muscle is given by the order, F > I > Br > Cl, the minimal concentrations being for NaF, M/64 to M/96; for NaI, M/32; for NaBr, M/16 to M/32; and for NaCl, M/16. This is connected, as Loeb points out, with the rapidity of passage into the muscle. He has recently shown (1919–20) that the same order holds for the influence of these ions on the rate of diffusion of water through collodion membranes.

Kruse (1919) has shown that a bromide-Ringer or Locke solution is inadequate to maintain rhythmicity in a perfused isolated mammalian heart for as long a time as a similar chloride solution, but claims that in the frog heart the bromide-Ringer solution is at least as effi-

cient as a chloride-Ringer solution. Macht and Hooker (1918) have shown that perfusion of the medulla of a mammal by a bromide solution stimulates the respiratory and cardio-inhibitory centers. Kruse considers therefore that the bromides are more or less irritating to most tissues of the body, but states that the central nervous system of the frog is not depressed by sodium bromide.

Stiles (1901) showed that isolated strips of frog esophagus are maintained in rhythm by bromide-Ringer solution. Prochnow (1911) has shown that the sodium halides increase the tonus of the uterus and the smooth muscle of arteries in the order, NaF, NaI, NaBr, the excitability being increased. Kruse found that bromides stimulated smooth muscle (intestine of dog or cat) slightly under normal conditions, and markedly when arrhythmic or fatigued.

Greisheimer (1919) found that M/8 sodium bromide is more toxic than M/8 sodium chloride as judged by the effect on the motor nerve of the frog.

Finckh (1921) has tested the effects of sodium halides on the isolated frog heart. In bromide-Ringer solution the heart continues beating for a long time, whence Finckh concludes that the chloride ion plays no peculiar rôle in this connection. When iodide-Ringer solution is substituted, harmful effects are observed which are attributed to the formation of elementary iodine. Nitrate-Ringer solution gives a similar result; here the toxic effect is explained by the possible formation of NO_2 ions.

Experimental.

We have determined the survival periods of frog heart and gastrocnemius-sciatic preparations (*Rana pipiens*) immersed in modified Locke solutions in which varying amounts of sodium chloride were replaced by the corresponding molecular concentrations of the sodium salts of the anions studied, fluoride, bromide, iodide, chlorate, iodate, and nitrate (Table I). In each set of experiments the solutions were made up with freshly distilled water, and the preparations, dissected as quickly as possible, were immersed in them in shallow vessels, so that the oxygen supply could be regarded as sufficient. The volume of solution, compared to volume of tissue, was large. From time to time the preparations were observed and tested. The times

were noted at which the hearts ceased to respond to electrical stimulation (the times at which normal heart beat ceased were irregular), and the muscles ceased to respond to such stimulation applied (*a*) directly, and (*b*) through their nerves. In all cases it was found that nerve tissue died shortly before the death of the corresponding muscle.

TABLE I.

Composition of Modified Locke Solutions.

	M/7.8 NaF	M/7.8 NaCl	M/7.8 NaBr	M/7.8 NaI	M/7.8 $NaClO_3$	M/7.8 $NaIO_3$	M/7.8 $NaNO_3$
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
NaF	5.38						
NaCl		7.50					
NaBr			13.20				
NaI				19.23			
$NaClO_3$					13.65		
$NaIO_3$						26.0	
$NaNO_3$							10.9
$NaHCO_3$	0.10	0.10	0.10	0.10	0.10	0.10	0.10
KCl	0.075	0.075	0.075	0.075	0.075	0.075	0.075
$CaCl_2$	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Glucose	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Water to	1,000 cc.						

It was observed very early in the experiments that a difference of a few degrees of temperature had a marked effect on the duration of life of the muscle-nerve preparations, in whatever solutions they were immersed. This is in agreement with previous observations of Cameron and Brownlee (1915–16) (Table II), while Gunn and Underhill (1914–15) have shown that temperature produces a similar effect in surviving mammalian tissue (intestine). Utilizing the fairly stable temperature conditions of the city water supply through the long winter here, it was possible to obtain observations throughout which the temperature remained fairly constant at 5°C. The shallow beakers containing the preparations were immersed in a large sink through which water from the city main flowed constant, and in most of the experiments the solution temperatures were thus kept constant to 5° ± 1°C. By selecting suitable times experiments at

TABLE II.

The Effect of Temperature.

Experimental Series No.	Number of frogs used.	Temperature.	Survival times.								
			Heart.			Muscle.			Nerve.		
			Maximum.	Minimum.	Mean.	Maximum.	Minimum.	Mean.	Maximum.	Minimum.	Mean.
100 per cent M/7.8 NaCl solutions.											
		°C.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
2	6	5	48.4	45.8	47.2	173.8	167.7	171.9	173.0	166.9	170.0
7	4	5	58.4	54.0	56.7	222.7	211.2	218.5	210.7	202.2	207.9
10	6	5	58.8	57.6	58.2	230.7	227.2	228.9	228.5	226.0	227.7
1	7	5–8	57.3	49.0	52.3	172.8	167.2	169.6	171.8	162.5	168.9
3	6	5–8	46.1	43.9	44.9	169.9	162.7	165.1	167.5	161.3	165.0
5	4	15–16	3.5	3.1	3.3	49.0	47.5	48.3	48.3	47.0	47.4
6	6	25	2.9	2.2	2.5	8.4	7.6	8.0	8.1	7.2	7.7
Cameron and Browniee.	6	29	Less than 1.			Less than 6.					
	3	32									
	6	36				Less than 2.					
	6	39				Less than 1.					
100 per cent M/7.8 NaBr solutions.											
1	7	5–8	33.4	30.4	32.3	123.9	121.8	122.1	122.7	120.9	121.8
5	4	15–16	2.4	2.2	2.2	35.0	34.3	34.8	34.5	34.0	34.2
6	6	25	1.7	1.6	1.7	7.1	6.8	6.7	6.8	6.2	6.4
100 per cent M/7.8 NaI solutions.											
1	7	5–8	8.1	7.5	7.6	46.4	44.5	45.7	45.5	43.1	44.4
5	4	15–16	0.87	0.77	0.83	11.4	10.3	10.8	10.7	10.2	10.4
6	6	25	1.12	0.75	0.97	4.1	3.3	3.7	3.8	3.2	3.5
100 per cent M/7.8 NaF solutions.											
1	7	5–8	0.18	0.12	0.13	1.67	1.25	1.43	1.33	0.95	1.25
5	4	15–16	0.15	0.13	0.14	0.65	0.52	0.57	0.57	0.47	0.52
6	6	25	0.13	0.10	0.12	0.48	0.37	0.42	0.45	0.32	0.37
100 per cent M/7.8 $NaClO_3$.											
4	6	5	20.0	18.2	19.3	78.5	74.7	76.3	77.3	73.2	75.2
5	4	15–16	1.8	1.6	1.7	14.9	14.2	14.7	14.2	13.5	14.1
6	6	25	1.5	1.1	1.3	4.9	4.2	4.5	4.5	3.8	4.2
100 per cent M/7.8 $NaNO_3$.											
4	6	5	14.6	12.7	13.7	61.8	57.8	60.3	60.5	56.7	59.2
5	4	15–16	2.1	1.9	2.0	18.9	18.2	18.7	18.7	18.0	18.3
6	6	25	2.0	1.7	1.8	5.0	4.7	4.8	4.7	4.3	4.5

TABLE III.

Effect of Halide Mixtures.

Experimental Series No.	Number of frogs used.	m/7.8 NaX	m/7.8 NaCl	Temperature.	Survival times.								
					Heart.			Muscle.			Nerve.		
					Maximum.	Minimum.	Mean.	Maximum.	Minimum.	Mean.	Maximum.	Minimum.	Mean.
Experiments with NaBr.													
		per cent	*per cent*	°C.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
1	7	100	0	5–8	33.4	30.4	32.3	123.9	121.8	122.1	122.7	120.9	121.8
2	6	50	50	5	36.9	34.6	35.5	143.7	139.6	142.4	142.8	138.4	121.2
3	6	25	75	5–8	46.1	43.8	44.9	169.9	162.7	165.1	167.5	161.3	165.0
7	4	12	88	5	46.6	43.7	45.3	194.7	191.2	193.0	186.2	182.7	184.4
8	4	5	95	5	51.6	50.0	50.7	202.5	197.0	200.1	192.6	190.7	191.4
7	4	0	100	5	58.4	54.0	56.7	222.7	211.1	218.5	210.7	202.2	207.9
Experiments with NaI.													
1	7	100	0	5–8	8.1	7.5	7.6	46.4	44.5	45.7	45.5	43.1	44.4
2	6	50	50	5	12.3	11.5	12.0	64.7	60.9	62.9	63.7	60.2	61.8
3	6	25	75	5–8	15.8	14.6	15.1	74.0	67.8	70.5	72.7	66.5	69.0
7	4	12	88	5	29.2	27.3	28.2	123.2	120.7	121.8	112.3	111.2	111.7
8	4	5	95	5	32.7	31.2	32.2	131.6	130.7	131.2	122.6	120.7	121.5
9	7	2.5	97.5	5	42.7	40.9	42.2	171.0	168.7	169.7	162.7	159.6	160.7
10	6	5	95	5	34.2	34.0	34.1	131.6	130.3	130.7	131.2	129.9	130.4
10	6	4	96	5	38.2	37.4	38.0	140.0	139.0	139.5	139.7	138.8	139.5
10	6	3	97	5	41.7	41.0	41.5	154.0	153.0	153.7	153.7	152.7	153.3
10	6	2	98	5	47.5	46.2	46.8	189.3	188.7	188.7	188.7	188.2	188.3
10	6	1	99	5	52.5	52.0	52.3	213.5	212.3	212.6	212.7	211.7	212.2
10	6	0	100	5	58.8	57.6	58.2	230.7	227.2	228.9	228.5	226.0	227.7
Experiments with NaF.													
1	7	100	0	5–8	0.18	0.12	0.13	1.67	1.25	1.43	1.33	0.95	1.25
2	6	50	50	5	1.9	1.2	1.5	6.3	4.7	5.3	5.8	4.1	4.8
3	6	25	75	5–8	4.6	2.7	3.4	8.4	6.3	6.9	7.4	5.8	6.4
7	4	12	88	5	12.4	11.9	12.0	22.7	21.7	22.7	20.4	19.4	20.1
8	4	5	95	5	25.0	24.7	24.8	49.7	48.7	49.1	40.7	39.0	40.0
7	4	0	100	5	58.4	54.0	56.7	222.7	211.2	218.5	210.7	202.2	207.9
Experiments with $NaClO_3$.													
4	6	100	0	5	20.0	18.2	19.3	78.5	74.7	76.3	77.3	73.3	75.2
2	6	50	50	5	21.7	20.4	21.0	82.5	79.0	81.1	81.9	78.6	80.1
3	6	25	75	5–8	25.4	23.5	24.5	89.8	82.8	86.8	87.6	81.7	85.1
7	4	12	88	5	33.0	32.0	32.5	149.2	146.7	147.7	139.7	136.8	138.6
8	4	5	95	5	42.1	40.8	41.3	161.0	159.7	160.5	151.6	149.8	150.7
7	4	0	100	5	58.4	54.0	56.7	222.7	211.2	218.5	210.7	202.2	207.9

TABLE III—*Continued.*

Experimental Series No.	Number of frogs used.	m/7.8 NaX	m/7.8 NaCl	Temperature.	Survival times.								
					Heart.			Muscle.			Nerve.		
					Maximum.	Minimum.	Mean.	Maximum.	Minimum.	Mean.	Maximum.	Minimum.	Mean.
Experiments with $NaIO_3$.													
		per cent	*per cent*	°C.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
10	6	10	90	5	24.7	24.0	24.3	96.6	93.4	95.0	95.0	92.6	94.0
10	6	5	95	5	31.6	30.0	30.7	121.8	120.0	121.5	121.5	119.7	121.2
10	6	4	96	5	34.0	33.0	33.2	134.0	133.4	133.7	133.6	133.1	133.4
10	6	3	97	5	37.7	37.2	37.5	144.7	143.8	144.3	144.2	143.5	143.8
10	6	2	98	5	43.0	42.0	42.5	158.8	157.6	157.8	158.4	157.0	157.3
10	6	1	99	5	49.7	49.2	49.5	169.7	167.0	168.6	168.4	167.2	168.2
10	6	0.5	99.5	5	54.7	53.6	54.5	179.0	178.4	178.7	178.2	177.6	177.7
10	6	0.1	99.9	5	56.7	56.3	56.7	218.4	217.2	217.7	217.8	216.8	217.3
10	6	0	100	5	58.8	57.6	58.2	230.7	227.2	228.9	228.5	226.0	227.7
Experiments with $NaNO_3$.													
4	6	100	0	5	14.6	12.7	13.7	61.8	57.8	60.3	60.5	56.7	59.2
2	6	50	50	5	19.4	17.4	18.2	71.2	65.7	68.6	69.4	64.7	67.3
3	6	25	75	5–8	23.6	19.7	21.4	73.7	70.9	72.3	72.0	69.4	70.6
7	4	12	88	5	29.6	28.2	28.7	136.3	134.0	134.6	125.8	123.1	125.2
8	4	5	95	5	35.7	35.0	35.3	140.7	138.7	139.5	131.2	130.0	130.5
9	7	2.5	97.5	5	38.9	36.7	37.6	150.7	147.5	149.4	140.7	138.1	139.7
10	6	5	95	5	38.5	37.7	38.0	140.0	139.0	139.7	139.7	138.7	139.4
10	6	4	96	5	40.7	39.9	40.5	151.4	150.0	150.5	151.2	149.7	151.2
10	6	3	97	5	42.5	41.5	42.2	160.4	160.0	160.1	160.2	159.7	159.7
10	6	2	98	5	43.6	43.2	43.5	172.2	171.1	171.5	171.7	170.7	171.3
10	6	1	99	5	49.2	48.9	49.1	187.2	186.3	186.9	186.7	185.7	186.6
10	6	0.5	99.5	5	55.1	54.8	55.0	200.2	199.4	199.9	199.7	198.9	199.6
10	6	0.1	99.9	5	56.0	55.1	55.7	213.7	212.4	212.9	213.3	212.1	212.5
10	6	0	100	5	58.8	57.6	58.2	230.7	227.2	228.9	228.5	226.0	227.7

other temperatures were made, with approximately the same degree of variation. In this way the effect of temperature has been ascertained fairly accurately. These temperature results are shown in Table II.

The modified Locke solutions employed (Table I) were equimolecular. Mixtures in known proportions gave solutions in which defi-

nite percentages of sodium chloride were replaced by the corresponding molecular amounts of the other anions. The actual molecular concentration of halide was therefore always M/7.8. With the degree of dilution employed, differences in degree of ionization can be disregarded. Table III shows the effects of such mixtures, for each of which the molecular percentage of the anion studied is indicated as "NaX."

The chemicals used were of the following degree of purity: sodium chloride, sodium hydrogen carbonate, potassium chloride, and calcium chloride, Kahlbaum, "zur Analyse" (pre-war manufacture); sodium fluoride, glucose, Merck's pure; sodium bromide, sodium iodide, sodium chlorate, sodium iodate, and sodium nitrate, Merck's U.S.P.

In all cases the animals were weighed before the experiment. Assuming that the weights of the tissues used were proportional to total body weight, there was no proportionality between weight of tissue and duration of life. The extreme weight limits in all experiments were 30 and 67 gm. In each experiment the extreme difference was much smaller. Almost all the animals were male.

DISCUSSION.

Our results show a fair degree of constancy, as exhibited both by the figures for the same conditions in different experimental series (the experiments in any particular series were commenced at approximately the same time and therefore those in one series are somewhat more accurately comparable) and the extreme variations obtained under any one set of conditions. The actual differences observed in the three different experiments with chloride-Locke solution at 5°C. are probably in part referable to different temperature fluctuations within the slight limits mentioned (5 ± 1°C). The maximum survival time of frog muscle in chloride-Locke solution was 230.7 hours; of frog nerve, 228.5 hours; and of frog heart muscle, 58.4 hours. These figures appear to be somewhat greater than any previously recorded, the difference being almost certainly due to temperature.

The period of survival decreases with increase of temperature in all solutions. The results for frog muscle are shown in Fig. 1. Those for heart and nerve give similar curves. (The mean temperature for

Experimental Series 1 and 3 has been taken as 6.5.) The halide curves for muscle are logarithmic; those for nitrate and chlorate do not show this relationship. The decrease is probably due to the combined effect of temperature *per se* (especially evident at higher temperatures and due presumably to an acceleration of all metabolic processes in the tissue) and of temperature in increasing the toxic effect of the foreign ion.

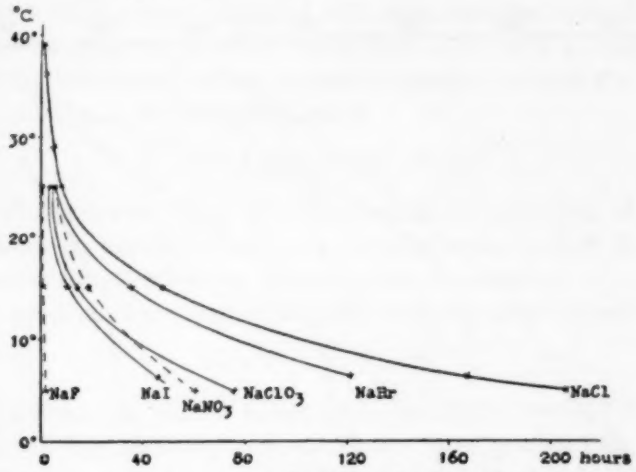

FIG. 1. Effect of temperature on the survival period of frog muscle immersed in various Locke solutions.

Introduction of any foreign ion into the Locke solution, under constant temperature conditions, decreases the survival period. This must be regarded as due to toxicity of the foreign ion. The greatest relative toxic effect appears to be produced by an initial slight replacement of chloride ions. Further replacement does not produce such a relatively great effect. This is shown by the curves for frog muscle in Fig. 2. Nerve and heart data yield similar curves.

It is therefore apparent that even small traces of such foreign ions (including bromide and iodide) are distinctly, though but slightly toxic. This is not in agreement with Finckh's conclusions, but is in general agreement with Kruse's results. Since, in solutions containing only a small percentage of replacements by the foreign ions, the survival period is in most cases at least several days, it seems un-

likely that the controlling factor is the relative permeability of the cell membrane to these ions. The important factor is probably a direct chemical action of the foreign ion on the cell protoplasm or some part of it.

Finckh has shown that very similar results are obtained with iodide and nitrate-Ringer solutions. Our results are in agreement, but it seems to us, on chemical grounds, since this parallel toxicity

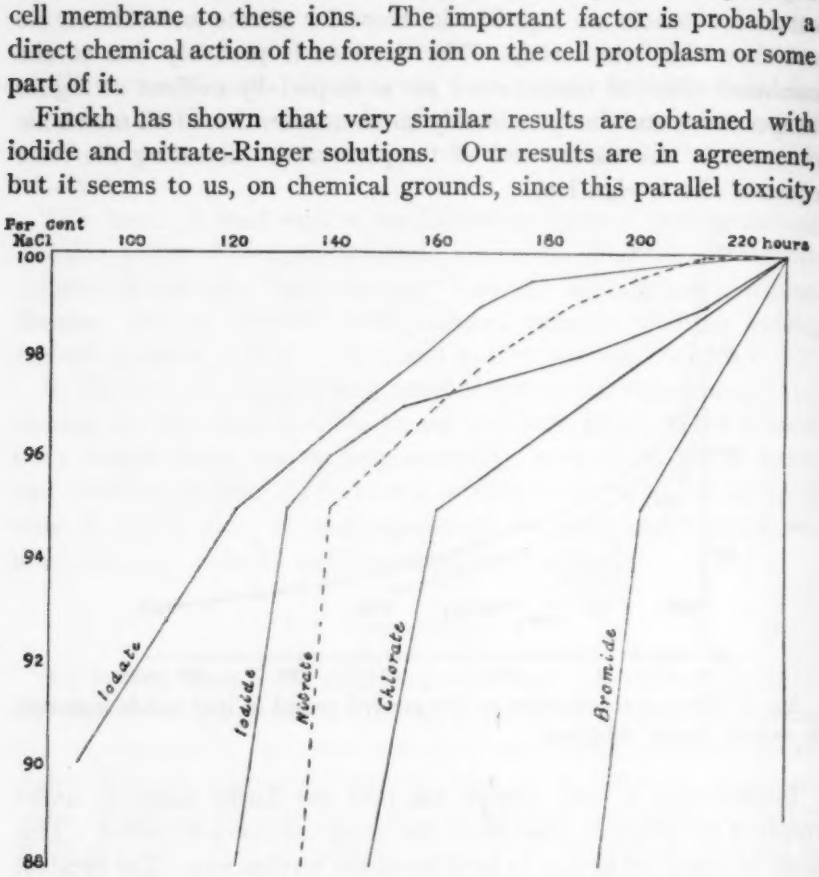

FIG. 2. Effect of substitution of chloride in Locke solutions by other anions on the survival period of frog muscle.

persists in solutions in which the I and NO_3 concentrations are very slight, that it is extremely unlikely that Finckh's conclusion is correct that the toxicity can be traceable to the formation of elementary iodine and of NO_2 ions.

Our results show (Fig. 2) a distinct difference of action in solutions in which 2 and 3 per cent of chloride ion is replaced by iodide and nitrate. For the greater replacement iodide is more toxic than nitrate;

for the lesser iodide is less toxic. This sudden decrease in degree of toxicity with greater dilution seems to be in agreement with the non-observance of distinct toxic effects after administration of small doses of iodide to mammals. There would appear to be a similar abnormal decrease for still smaller iodate concentrations, as contrasted with nitrate-chloride mixtures (Fig. 2). Nevertheless, even very slight traces of any of the foreign ions that we have tested exhibit a distinct toxicity, when compared with pure chloride-Locke solution.

For Locke solutions in which more then 5 per cent of chloride is replaced by the corresponding molecular concentration of the foreign ion, the toxicity is in descending order

$$F > IO_3 > I > NO_3 > ClO_3 > Br > Cl$$

This is in agreement with the effectiveness in producing rhythmic contractions in muscle (Loeb); the inverse order is that found by Biedermann for effectiveness as coenzymes of amylases.

When less than 2 per cent of the chloride is replaced the order is

$$IO_3 > NO_3 > I > Br > Cl$$

These results can almost certainly be considered to hold for body fluids circulating normally and containing these foreign ions.

The much greater relative toxicity of fluoride solution under all conditions is in agreement with the marked biochemical differences of fluoride and the other halide ions.

M/7.8 chlorate-Locke solution produced a regular series of rhythmic contractions (about 35 per minute) in muscle. M/7.8 iodide-Locke solution produced a similar effect; the rate was somewhat faster. Fluoride produced a continuous fibrillation. The chlorate and iodide effects were not observed with muscle of curarized frogs, though the fluoride fibrillation persisted. No such effects were observed with the other anions studied.

This research forms part of a series relating to the biochemistry of iodine. Part of the expenses were defrayed by a grant from The Chemical Society (of London) to whom we tender our thanks.

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SOME EFFECTS OF RADIUM RADIATIONS ON WHITE MICE.

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It is well known that radium rays, as well as x-rays, are capable of affecting the living cell. Bergonié and Tribondeau[1] from extensive experimental studies made the generalization[2,3] that: "Immature cells and cells in an active state of division are more sensitive to the x-rays than are cells which have already acquired their fixed adult, morphological or physiological characters."[2] Subsequent observations have resulted in the extension of this generalization to the effect of radium radiations as well. For this reason the mice of the experiments described in the present paper were exposed to radium rays soon after birth and during the period of rapid growth.

Experimental.

The body weight of mice at birth and their subsequent rate of growth are influenced by many factors. It is essential, therefore, that control experiments be carried out with animals as nearly as possible like those treated; and that external conditions be identical for both groups during the period of observation. The age, physical condition, and body weight of the mother, the length of the gestation period, the size of the litter, and its position in the litter series affect the inital weight of mice and their growth. Accordingly, a litter of young was divided into two nearly equal groups of both sexes; one to be exposed to radiation and the other to be used as control. The method devised

[1] Bergonié, J., and Tribondeau, L., *Compt. rend. Acad.*, 1906, cxliii, 983.

[2] Colwell, H. A., and Russ, S., Radium, x-rays, and the living cell, London 1915, 253.

[3] Béclère, M., Cottentot, P., and Laborde, S., Radiologie and radiumtherapie, Paris, 1921, 223.

by Jackson[4] for albino rats was used to determine the sex of the new born mice. Any underdeveloped animals were discarded, but otherwise the litter was divided into the two groups at random. In this way individual inequalities would be divided fairly evenly between the two groups. It was found, in fact, that the average weight of the mice of one group from day to day was very nearly the same as for the other group, when neither was treated with radium.

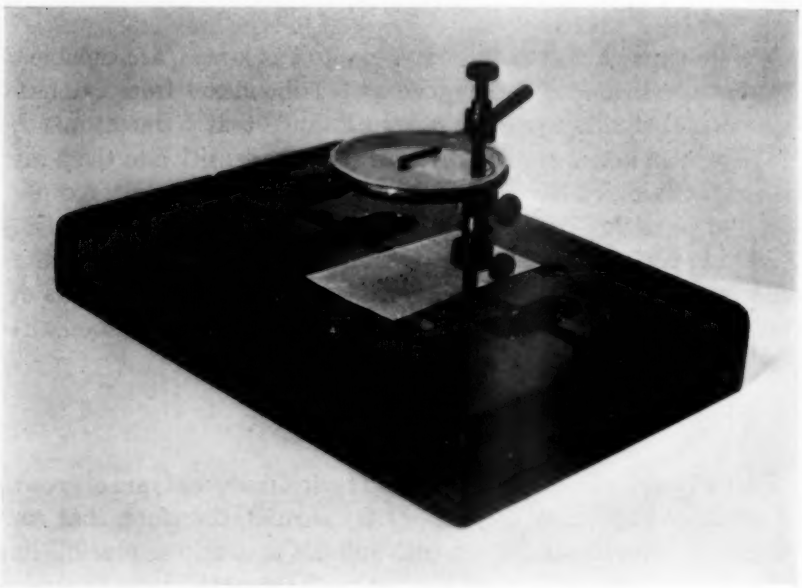

FIG. 1.

The apparatus of Fig. 1 was used to irradiate the mice, one at a time. The new born animal was placed in a cavity slightly larger than itself and was covered with a very thin sheet of mica. In this manner the mouse remained in a fixed position during the treatment, with its back touching the mica. A capillary glass tube 10 to 14 mm. in length, and about 0.5 mm. in external diameter, containing radium emanation, was placed on another sheet of mica which was rigidly supported at a distance of 2 cm. from the animal. The holder was

[4] Jackson, C. M., *Biol. Bull.*, 1912, xxiii. 171

TABLE I.

Experimental conditions, and doses of radiation.

Experiment No.	No. of mice radiated.		No. of controls.		Part of body radiated.	Distance between animal and tube.	Duration of daily exposure.	Strength of emanation tube used.												Total dose.
	Male.	Female.	Male.	Female.				1st day.	2nd day.	3rd day.	4th day.	5th day.	6th day.	7th day.	8th day.	9th day.	10th day.	11th day.	12th day.	
						cm.	*min.*													*millicurie hours*
1	1	2	2	2	Back.	2	5	4.3	6.0	5.2	4.0	3.8								1.9
2	2	2	2	2	"	2	5	5.4	4.5	6.7	6.7	5.6								2.4
3	2	2	2	1	"	2	5	5.0	6.0	5.0	5.0	4.5	7.5	4.8	5.0	4.0	3.4	2.9	6.0	4.9
4	1	2	1	1	Head.	2	5	10.5	9.0	7.6	6.4	5.4	8.0	6.7	8.0	6.9	7.5	6.3		6.8
5	4	2	2	3	Back.	2	5	14.5	12.2	14.2	11.0	11.0	11.0	10.5	11.0	10.0	10.0	9.0	12.0	11.5
6	1	1	1	3	Head.	2	5	27	23	29	30	32	33	31	26	32				21.9
7	1	3	1	3	"	2	5	30	33	28	30	33	31	28	30	28	27	23		26.8
8	2	2	1	1	Back.	2	5	48	48	48	53	45	38	54	45					31.6
9	2	2	1	2	"	2	5	65	40	34	29	50	50	50	62	58				36.5

adjusted so that the tube was either directly over the central dorsal region or over the head of the animal. The time of exposure was always 5 minutes. The amounts of emanation employed and the number of treatments given on successive days are shown in Table I.

To identify the controls from the radiated mice in the same litter a small piece of tail was clipped off from the specimens of one group. The mice of each group were weighed together for the first time within 12 hours of the time of birth, and then every 24 hours until the 21st day, when the young were suckled. From this time on the mice were weighed individually about once a week. For any group the average weight for one mouse was calculated. A careful record was kept of the physical condition, body weight, and hair development for the different mice; also the time when they opened their eyes or reached sexual maturity, and the duration of life.

The biological effect of radium radiations is related to the amount of radiant energy absorbed by the tissue. This depends on various factors, of which the quality of radiation used and the distance of application are the most important ones. At the present time there is no satisfactory unit in which to express the dose of radiation administered. In these experiments radium emanation enclosed in capillary glass tubes of a definite size was used as the source. The quality of the radiation employed, mainly β-rays, therefore, was always the same, and the animal was placed at a distance of 2 cm. from the emanation tube for every treatment. Under these conditions the dose depends only on the amount of emanation in the tube and the duration of the application. The former is given in millicuries and the latter can be expressed in hours; the product of the two (millicurie hours) in this case may be taken to represent the relative doses. It should be noted in this connection that in each case the millicurie hour dose appearing in Table I is the total administered during the whole period of treatment in fractional daily doses.

The typical effects of different doses are shown graphically in Fig. 2. Here the body weights of the radiated mice and their respective controls are given for a period of 8 weeks, this being the time during which the maximum radium reaction is likely to occur. It will be seen that a dose of 2.4 millicurie hours, fractionally applied, in the manner previously described, accelerated the growth of the mice.

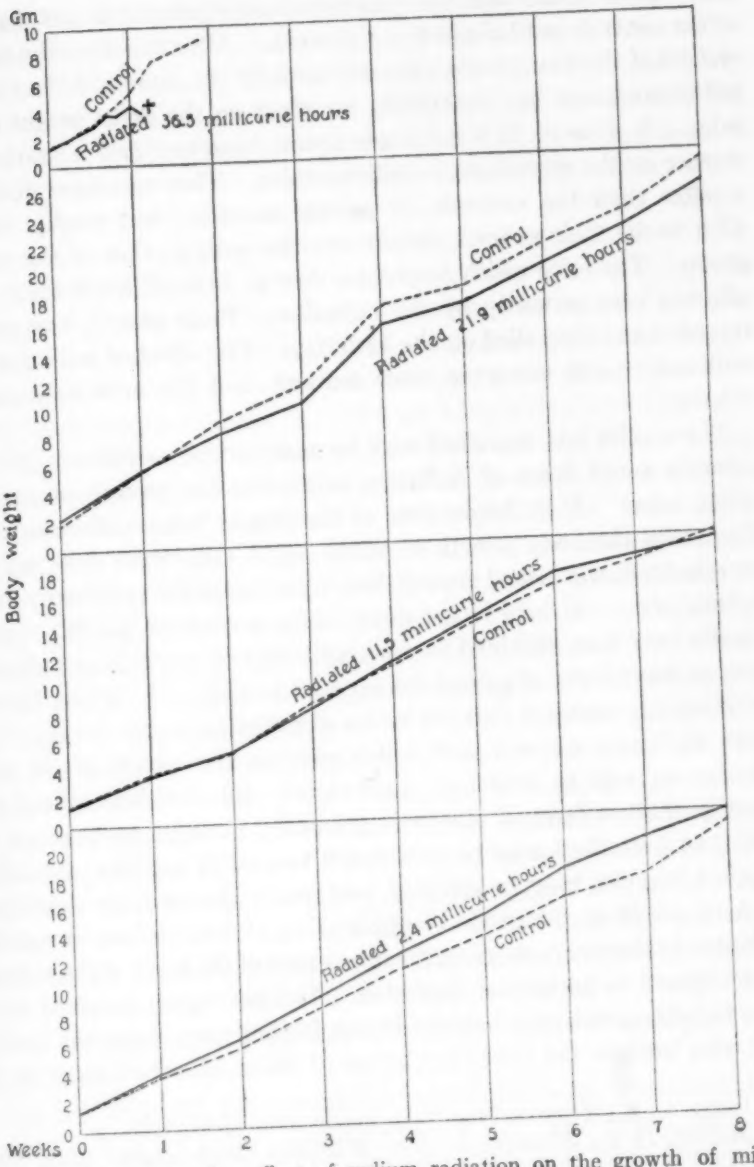

FIG. 2. Charts showing effect of radium radiation on the growth of mice. Source of radiation, one tube of radium emanation, 14 mm. long and 0.5 mm. in diameter. Distance of source, 2 cm. No metallic filter. Divided doses are shown in Table I.

The weight of the radiated mice remained distinctly larger than that of the controls until about the 27th week. After this time the average weight of the two groups was substantially the same. A dose of 11.5 millicurie hours had practically no effect on the body weight of the mice. A dose of 21.9 millicurie hours, however, had a marked influence on the growth of the radiated mice. They remained distinctly smaller than the controls for several months, but finally (in the 45th week) their average weight was the same as that of the control group. The mice which received a dose of 31.6 millicurie hours were affected very seriously by the radiation. Their growth was greatly retarded and they died on the 12th day. The effect of a dose of 36.5 millicurie hours was even more marked, and the mice died on the 9th day.

The results just described may be summarized as follows: (*a*) Sufficiently small doses of radiation accelerate the growth of suckling white mice. (*b*) A larger dose of the proper value will have no influence on the body growth of mice. (*c*) A still larger dose, up to a certain limit, will retard growth, but the animals will eventually attain normal size. (*d*) Still larger doses cause premature death. Similar results have been obtained before in experiments on seeds and plants,[5,6] also on lower forms of animal life exposed to x-rays.[7,8] From these it is commonly assumed that the action of radiation on the living cell follows the same general law[9] which governs the action of all anesthetics, as well as chemical, mechanical, and electrical stimulants; that is, if some form of energy is gradually brought to bear on the cells, at first they may be stimulated to greater activity, then their normal function may be arrested, and finally they may be destroyed.

As a result of the mode of application of the radium emanation adopted in these experiments, the upper part of the body of the animals was exposed to an intense radiation. The lower part received much less radiation, not only because it was farther away from the source, but also because the overlying layers of tissue absorbed most of the

[5] Gager, C. S., Memoirs, New York Botanical Gardens, 1908, iv.

[6] Molisch, H. C., *Sitzungsb. k. Akad. Wissensch. Math-naturw.*, Wien, 1912, cxxi, 121.

[7] Davey, W. P., *J. Exp. Zool.*, 1917, xxii, 573.

[8] X-ray Studies, General Electric Company, Schenectady, 1919, 255, 267.

[9] Christen, Th., *Strahlentherapie*, 1919, lx, 590.

β-rays. The emanation tube was placed parallel to the length of the animal to obtain a more even distribution of the radiation. Nevertheless it should be expected that the skin directly under the tube would receive more radiation, especially on account of the curvature of the body. Accordingly, when the dose was sufficiently large, marked local effects were produced on the backs or heads of the animals. The results may be summarized as follows: There were no visible changes in the skin and the hair growth of mice exposed to doses of 1.9, 2.4, and 4.7 millicurie hours. A dose of 6.8 millicurie hours retarded the development of lanugo hair directly opposite the emanation tube, and produced a skin erythema. The growth of hair, however, became normal on the 15th day after birth. Larger doses of radiation produced more pronounced effects. The mice irradiated with 11.5 millicurie hours had no hair on their backs on the 8th day. The hairless area was completely filled with new hair on the 14th day, but its retarded growth could be noticed up to the 30th day. The animals exposed to 21.9 millicurie hours showed wrinkled and reddened scalps on the 10th day. The radium reaction on the skin became worse up to the 15th day, when it was at its height. The skin was dry, scaly, wrinkled, and hemorrhagic. The eyes were inflamed and almost closed; the ears red, swollen, and underdeveloped. The healing process began at this time and progressed slowly. The effects from 26.8 millicurie hours were similar to these but more severe. On the 13th day the mice were unable to run normally, but could get about only with great difficulty. However, the animals gradually regained their health and vigor. A narrow strip of hairless skin, over which the emanation tube had been applied at a distance of 2 cm. was still visible at the end of the 616 day. Doses of 31.6 and 36.5 millicurie hours caused the mice to die on the 12th and 9th days respectively. Their hind legs were completely paralyzed the day before death. The viscera were very much undersized, and the brains hemorrhagic and congested.

The age at which the normal mice open their eyes is variable, but generally it is 13 days. Among mice of the same litter, however, there is little irregularity. Female mice often open their eyes a few hours earlier than the males. A record was kept of the age of the individual mice at the time they opened their eyes, and the averages,

for each group of controls and radiated mice, are given in Table II. It will be seen that doses below 11.5 millicurie hours had no apparent effect on the time when the radiated mice opened their eyes. But the mice receiving larger doses of radiation opened their eyes from 12 to 24 hours earlier than their respective controls. Similar effects were

TABLE II.

The Effect of Radium Radiations on Opening of Eyes in White Mice.

Experiment No.	Group.	No. of mice radiated.		Average age at which eyes opened.
		Males.	Females.	
				days.
1	Controls.	2	2	13.1
	1.9 millicurie hours.	1	2	13.0
2	Controls.	2	2	13.9
	2.4 millicurie hours.	2	2	14.0
3	Controls.	2	1	13.2
	4.9 millicurie hours.	2	2	13.1
4	Controls.	1	1	12.0
	6.8 millicurie hours.	1	2	12.0
5	Controls.	2	3	13.7
	11.5 millicurie hours.	4	2	13.2
6	Controls.	1	3	13.0
	21.9 millicurie hours.	1	1	12.0
7	Controls.	1	3	12.9
	26.8 millicurie hours.	1	3	12.2

noted by Tribondeau and Belley,[10] who x-rayed the eyes of young kittens and found that the radiated eyes opened always some hours before the controls.

Many experimenters have noted that radium and x-rays can bring about sterility in animals. The literature is abundant,[11] but in many

[10] Tribondeau, L., and Belley, G., *Arch. Med.*, 1907, xv, 907.

[11] Albers-Schönberg, *Münch. med. Woch.* 1903, l, 1859. Frieben, *Münch. med. Woch.*, 1903, l, 2295. Philipp, *Fortschr. Geb. Röntgenstrahlen*, 1904, viii, 114. Halberstædter, L., *Berl. klin. Woch.*, 1905, xlii, 64. Bergonié, J., Tri-

cases the doses of radiation which caused sterility either are not given at all or they are given incompletely. The marked response to radiation which the reproductive cells exhibit can be explained by Bergonié and Tribondeau's generalization, already referred to. In the experiments of this paper, the effect of radiation on the reproductivity of the mice was also investigated. For this purpose the radiated animals were mated with normal mice about the 35th day after birth. One radiated male was mated with two normal females of about the same age. The animals were kept in the same cage until both females had produced at least three litters of young. Two radiated females were mated with two normal males, and they were not separated until the females had reached the age at which they normally cease to breed. In general, the young were separated from the mother soon after birth. In the case of the mice of Experiments 1 and 4 the young of one litter in either experiment were allowed to remain with their mother until weaned. These mice and succeeding generations were kept under observation to determine whether radiation affected the offspring of the radiated mice.

The results of the breeding tests were as follows: All the males of Experiments 1 to 7, receiving doses of 1.9 to 26.8 millicurie hours, were fertile. (Those of Experiments 8 and 9 succumbed to doses of radiation larger than 26.8 millicurie hours before they reached sexual maturity.) On the other hand, with the exception of the mice of Experiments 1 to 4, all the radiated females were rendered permanently sterile. Those of Experiments 1 and 4 were fertile. This shows that a dose of 1.9 millicurie hours (Experiment 1) applied as already explained (Table I) was not sufficient to sterilize female white mice. However, with one exception (Experiment 4) larger doses were effective. The apparent anomaly when a dose of 6.8 millicurie hours was used (Experiment 4) is due to the fact that in this case the emanation tubes were placed directly over the heads of the animals (Table I). Thus the ovaries were at a greater distance from the radioactive source, and they also were better protected from the radiation by a greater thickness of tissue in the path of the rays. In Experiment 6, the dose, 21.9 millicurie hours, was sufficient to cause sterilization, even though

bondeau, L., and Récamier, D., *Compt. rend. Soc. biol.*, 1905, xii, pt. 1, 284.
Brown, T., and Osgood, A. T., *Am. J. Surg.*, 1904–05, xviii, 179.

the mode of application was the same as in Experiment 4. The controls of either sex were all fertile. The first litter of young from both radiated and non-radiated female mice was obtained between the 60th and 78th day after birth. The radiated males reached sexual maturity at about the same time as the controls. The young born from the radiated mice grew normally and in turn produced normal

TABLE III.

Effect of Radium Radiations upon Longevity of White Mice.

Experiment No.	Group.	No. of mice used.	Days after birth.	No. of mice alive.	Days after birth.*	No. of mice alive.
1	Control.	4	384	4	587	2
	1.9 millicurie hours.	3		3		1
2	Control.	4	327	0		
	2.4 millicurie hours.	4		0		
3	Control.	3	485	2	602	1
	4.9 millicurie hours.	4		3		1
4	Control.	2	186	2	Discontinued.	
	6.8 millicurie hours.	3		3		
5	Control.	5	502	5	618	3
	11.5 millicurie hours.	6		5		3
6	Control.	4	404	4	607	1
	21.9 millicurie hours.	2		2		2
7	Control.	4	500	2	616	2
	26.8 millicurie hours.	4		3		2

* Final observation made November 20, 1921.

young, judging from their general appearance, weight, and reproductive power.

Davey[7] has reported some experiments with *tribolium confusum* in which by small doses of x-rays he was able to prolong the average life of the beetles. He used a very large number of specimens, and the statistical results are quite reliable. From our experiments, however, no definite conclusions can be reached as to the effect of radia-

tion on the longevity of white mice. The number of animals used was too small to apply statistical methods, and some of the mice are still living. It is interesting to note, however, that even those mice which showed severe local and constitutional effects from the radiation apparently did not have their lives shortened appreciably. Some were a little undersized but otherwise apparently normal on the 618th day. Table III shows the number of mice still living at two different periods in the course of the experiments.

DISCUSSION AND SUMMARY.

It has been estimated[12] that 92 per cent of the total radiation emitted by radium in equilibrium with its subsequent products is given off in the form of α-rays. This, however, cannot be utilized when the source is enclosed in an ordinary container, because the α-rays are absorbed completely by even a small thickness of glass. About 3.2 per cent of the total radiation is emitted in the form of β-rays, and 4.8 per cent as gamma radiation. The effects produced on the radiated mice of these experiments were due mainly to the β-rays, which are easily absorbed by tissue. The γ-rays, being only slightly absorbed by organic matter, probably contributed very little to the observed effects.

It is interesting to correlate the different effects produced by the same dose of radiation. The mice which received a dose of 1.9 millicurie hours showed no local effects on the skin or hair. Neither females nor males were sterilized, and the time at which they opened their eyes or reached sexual maturity was not affected, as far as we could tell. The only difference noted between the radiated animals and the controls was in the body weight. This dose accelerated the growth of the young mice, that is, while initially of the same weight, soon after irradiation they became distinctly bigger than the controls, but finally the animals of each group had substantially the same average weight. That this variation in body weight should be accidental is unlikely, since it was observed also in the animals treated by a slightly larger dose (2.4 millicurie hours). The number of animals

[12] Rutherford, E. Radioactive subtances, and their radiations, Cambridge, 1913, 581.

(seven) which showed this effect is too small to prove conclusively the accelerating effect of small doses of radiation on the body growth of mice. But considering that similar results have been obtained by radiating plants[6,7] and beetles,[7,8] it is reasonable that the observed increase in weight might be attributed, at least in part, to the effects of radiation. Since this paper was first written Russ, Chambers, and Scott[13] have shown that small doses of x-rays accelerate the body growth of rats. In view of this additional evidence there can be little doubt that the increase in weight observed in our experiments was due to the radiation.

A dose of 2.4 millicurie hours applied over the backs of the animals produced no local skin effects, but it accelerated the growth of the mice as in the previous case. In addition it caused permanent sterilization of all the females. A similar result was obtained with 4.9 millicurie hours, except that the effect on the rate of growth was uncertain. A dose of 6.8 millicurie hours produced a definite but mild skin erythema and retarded the development of lanugo hair. But since in this instance the emanation was applied over the heads of the animals, the dose reaching the ovaries was not sufficient to cause sterilization, as already explained. No other definite effect was noted.

In connection with the sterilization of the females it should be noted that a dose of radiation which produced no visible skin changes was sufficient to cause permanent sterility. On account of the greater distance of the ovaries from the source of radiation as compared with that of the skin directly below the tube, and the depth of tissue which the rays had to traverse to reach the ovaries, the amount of radiation acting on the latter was much smaller than the amount falling on the skin. The radiation emitted by the emanation tube is reduced to about 50 per cent of its initial value after traversing 1 mm. of tissue. Still, while the skin was not visibly affected, the mice were sterilized. This shows that the ovaries are influenced very easily by radiation of this type. We can estimate the amount of radiation reaching the ovaries which is sufficient to cause sterility to be less than 25 per cent of the amount necessary to produce visible skin changes in the mice. It should be noted also that whenever sterility of the female mice was

[13] Russ, S., Chambers, H., and Scott, G. M., *Arch. Radiol. and Elect.*, 1921, xxvi, 128.

induced, it was permanent. Furthermore, those mice which were not rendered sterile by radiation were, as far as the experiments enable us to say, as prolific as the controls. Remembering that a dose of 1.9 millicurie hours had no apparent effect on the ovaries, while a slightly larger dose, 2.4 millicurie hours, caused permanent sterility, it might be concluded that it is not possible to produce temporary sterility by radiation. We know, however, that temporary sterility can be produced, at least when the animals are radiated at a later stage in their development. The mice in our experiments were radiated for the first time soon after birth, and it is not improbable that under these conditions temporary sterility cannot be obtained.

Large sublethal doses produced severe skin burns, retarded the body growth of the animals, but failed to sterilize the males. About one-third of the total skin area of the mice showed marked effects from the radiation. The animals were very sick for a time, and their growth was temporarily stunted. But nevertheless they recovered and finally became apparently normal except for the narrow hairless strip of skin which had been closest to the emanation tube. Only the females were rendered permanently sterile. The males did not show even temporary sterility when the doses of radiation were close to the lethal dose. While the testes of mammals are known to be very easily affected by radiation, still they are more resistant than the ovaries. In addition, in these experiments they were at a greater distance from the source of radiation than the ovaries, and they were better protected by the thicker layer of tissue in the path of the rays. The fact that no sublethal dose in these experiments sterilized the males shows that under the conditions of irradiation adopted the amount of radiation reaching the testes was not sufficient to affect them noticeably. If the source of radiation had been applied closer to the reproductive organs of the males, they would have been sterilized by millicurie hour doses much smaller than the lethal dose.

Some of the radiated animals were killed with ether, and macroscopic and microscopic examinations of the reproductive organs were made. The ovaries of the sterile females were generally atrophied and colored yellow. The normal histological structure was altered. The characteristic findings were the destruction of the Graafian follicles,

with absence of ovum cells. The testes and the epididymis of the radiated mice of the present experiment appeared macroscopically and histologically normal, with the presence of abundant spermatozoa. Owing to the method adopted for the irradiation of the mice, the testes were too far from the source of radiation, and too well protected by the intervening tissue to be definitely affected by the rays.

BANANA GEL.

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INTRODUCTION.

The change of a soluble sucrase preparation from bananas into an insoluble preparation was described in a previous paper.[1] This change was found to occur on dialysis against running tap water. The insoluble form showed many of the characteristics of a gel. In this paper, a more complete study of the factors influencing the formation of this gel will be presented together with some of its properties but without considering its possible connection with enzyme activity.

EXPERIMENTAL.

Methods.

The banana extract was prepared by passing the pulp of ripe bananas through a fine food-chopper, mashing it in a mortar with the requisite amount of water or sodium chloride solution, and filtering through paper in large funnels. Toluene was added before the filtration. The extract had a brown tinge. It had a pH value in the neighborhood of 5.0. Bananas in the same stage of ripeness were used as far as possible. Individual minor variations in the gelling properties of extracts were observed at times, just as the constituents of bananas may differ to small extents.

Collodion bags were used for dialysis. These were prepared in the usual way but were not standardized. However, the conditions for their preparation were kept uniform so that possible errors from this source need not be considered in the present connection. The volume of liquid in any one bag was always less than one-fourth the capacity of the bag, to allow for the increase in volume due to the dialysis.

[1] Falk, K. G., and McGuire, G., *J. Gen. Physiol.*, 1920–21, iii, 595.

Hydrogen ion concentrations were determined by use of indicators and suitable standard solutions.

The various salts which were used were either purified, or the impurities determined, since in the work to be described, minute amounts of certain substances were found to produce striking phenomena.

Results.

I. Dialysis of Banana Extract against Tap Water and against Distilled Water.

The results obtained in a large number of experiments in the dialysis of banana extracts may be summarized briefly in the following paragraphs. Essentially the same results were obtained whether the extracts were prepared with water or with molar sodium chloride solution. As a rule, in the preparations 1 part by weight of liquid was used with 4 parts of banana pulp.

1. Dialysis of extract against tap water.

(*a*) Gel formation began in 24 to 48 hours. With small volumes more rapid changes including formation of gel occurred than with large volumes.

(*b*) Volume increased about 100 per cent. Most of the increase occurred before gel formation began. Changing bags during the dialysis had no effect.

(*c*) After two or three days dialysis the liquid still had a pH of 5.0 to 5.5.

(*d*) Gel formation began on the inner walls of the bags.

(*e*) Continued dialysis caused the dark colored gel to flake or separate out, leaving the liquid clear and colorless. As the flakes were formed, the pH of the supernatant liquid was 5.0 to 5.5 at first, but after 4 to 6 days became that of the tap water (pH 7.0).

2. Dialysis of extract against distilled water.

(*a*) No gel formation at any time.

(*b*) Continued increase in volume. In one experiment an increase from 25 cc. to 178 cc. in 4 days was observed.

(*c*) After dialysis pH of liquid unchanged (about 5.0).

(*d*) Liquid became cloudy and light colored (perhaps because of dilution).

3. Dialysis of gel from 1 (e) against distilled water.

(*a*) Gel disappeared on continued dialysis (up to 6 days) against renewed distilled water.

(*b*) Increase in volume as in 2 (*b*) in every case.

(*c*) Liquid became cloudy and lighter colored as in 2 (*d*) if the gel disappeared.

4. Dialysis of liquid from 2 (d) against tap water.

(*a*) Cloudiness disappeared.

(*b*) No further increase in volume.

(*c*) Some flakes of gel separated.

Toluene was present in all these experiments. However, long continued shaking of the extracts with toluene produced no gel. Bubbling air through the extracts also did not result in gel formation.

Most of the experiments were carried out by dialyzing against running tap water or distilled water. Some experiments dialyzing against tap water or distilled water in large beakers gave the same results.

The difference in the behavior of the banana extract relative to the gel formation upon dialysis against tap water and distilled water, was evidently due to differences in the compositions of the two. The pH of the tap water was found repeatedly to be 7.0 to 7.2: that of the distilled water about 5.0. An average analysis of the inorganic constituents, as parts per million of the tap water was as follows:[2]

$CaCO_3$	24	NaCl	5
$MgCO_3$	5	KNO_3	1
$MgSO_4$	10	SiO_2	9

The distilled water was practically salt-free.

II. Dialysis of Banana Extract against Solutions of Definite Hydrogen Ion Concentrations and Salt Contents.

Banana extract was dialyzed for 48 hours at 10–15°C. in collodion bags against the following solutions contained in large beakers with the indicated results. Sodium hydroxide or hydrochloric acid were

[2] The analysis was obtained through the courtesy of Mr. Merritt H. Smith, Chief Engineer of the Department of Water Supply, Gas and Electricity, New York City.

used when necessary to produce the required pH values. That sodium chloride did not play a positive part in the gel formations was evident from the fact that the banana extracts in many experiments were prepared with sodium chloride solution.

When gel formation occurred, the volume increases were 100 per cent or less, when no gel formation occurred the volume increases ranged from 100 to 400 per cent in 48 hours. In a number of experiments, the pH of the outside liquid changed from 7.0 to 6.0 or even less. Whenever this occurred, no gel was formed inside the bags.

These results showed that for the formation of a gel from banana extract by dialysis against tap water, the presence of a calcium salt

Solutions.	pH of outside liquid.	Results.
$CaCO_3$ 0.00020 to 0.00024 M[3]	7.0	Gel.
$CaCO_3$ 0.00020 to 0.00024 M; phosphate buffer[4], 35 cc. : 2 liters	7.2	"
$CaCO_3$ Saturated, excess solid	7.0	"
$CaCO_3$ 0.00024 M	5.0	No gel.
Phosphate buffer,[4] 35 cc. : 2 liters	7.2	" "
$MgSO_4$ 0.00024 M	7.0	" "
$MgSO_4$ 0.00024 M	5.0	" "
$Ca(OH)_2$ Saturated, excess solid CaO	More alkaline than 10.0	Gel (white).

and an alkalinity corresponding to pH 7.0 or more were the important factors.

In order to determine whether the collodion bag as such played a part in the gel formation or whether possibly interfering substances were removed by dialysis, the following experiments, in which a number of different salts were added directly to banana extract at different hydrogen ion concentrations, were carried out.

III. Actions of Salts on Banana Extract at Definite Hydrogen Ion Concentrations.

No satisfactory quantitative methods for the comparison of gels, the readiness of their formation, and their chemical compositions,

[3] 20 to 24 parts per million.

[4] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, 76.

are at hand. In the following experiments, in comparing different gels, their consistency is taken to be the greater or smaller resistance to deformation, the ability to retain the shape of the vessel in which they were prepared, even when separated from it, etc. In any one series a satisfactory comparison can be made, but in different series carried out at different times, anything more than a rough qualitative study is difficult.

Since calcium salts were found to be involved in the gel formation in the dialysis experiments, a number of series of experiments were carried out in which solutions of calcium salts were added directly to banana extracts under different conditions without dialyzing.

The acidity of the mixture was the first determining factor. A banana-water extract (4 parts of banana pulp with 1 part of water and filtered in the usual way) gave no indication of gel formation at the pH of the juice (about 5.0) with 0.29 mg. of calcium, added as calcium oxide, or 0.22 mg. of calcium, added as calcium chloride, per cc., in 48 hours at 5°–10°C. The same extract at pH 7.5 with 0.04 mg. of calcium as calcium oxide added per cc. formed a firm gel in 5 minutes which retained the shape of the container. These results were confirmed repeatedly. It appeared to be impossible to obtain gel formation with calcium salts with solutions more acid than about pH 6.0. With solutions at pH 7.0 or more alkaline, gels were obtained readily. The greater the concentration of calcium the more rapid the formation of a firm gel, and *vice versa*. For example, with a calcium concentration of 0.03 mg. per cc. and the juice mentioned above, gel formation had only begun after 20 minutes, while a firm gel was present in 7 hours. With 0.02 mg. of calcium per cc. at 5° in 18 hours, definite gel formation had occurred, while with 0.01 mg., the gel formation was doubtful. Low temperatures favored the formation of the gel. While extracts obtained at different times differed slightly, the general conclusion that with calcium salts no gel formation at pH 6.0 and more acid, and gel formation at pH 7.5 and more alkaline, was found to hold in every case.

It is of interest to note that if the banana extract is boiled for a few minutes, some solid shreds forming, a gel could not be obtained either by dialysis against tap water or at pH 7.5 by the addition of calcium salts.

These results are of importance in connection with the preparation of banana extract and subsequent treatment of the latter. As described previously, the banana extract was prepared with sodium chloride solution for a number of experiments. It was found necessary to purify the sodium chloride, since a number of samples of the latter were found to contain small quantities of calcium salts. If the calcium was not removed, on bringing the extract to pH 7.0 or more alkaline condition for various purposes, gel formation would take place very rapidly. Similarly, if sodium hydroxide which contained calcium as impurity, was used for neutralization, gel formation might occur. The results described above were therefore obtained by neutralization with ammonium hydroxide. An added complication was found in the fact that the banana itself contains very small amounts of calcium salts[5] which may appear in the extracts. Long standing (48 hours or more) in solutions of water extract brought to pH 7.0 with ammonium hydroxide resulted in the formation of some gel presumably from this cause.

Sodium chloride extracts showed smaller gelling actions with calcium salts than did water extracts. Thus, with two banana extracts, one prepared with water, the other with sodium chloride solution, treated with 0.02 mg. of calcium in the form of calcium chloride per cc. under the same conditions, formed a firm gel in the first case in 10 minutes, and very little gel in the second.

A comparative study of the gelling actions of certain salts was made. Two series were run, one at pH 5.0, and the other at pH 7.5. To 30 cc. portions of banana-water extract were added 0.5 cc., 1.0 cc., and 1.5 cc. of 0.044 M solutions of calcium chloride, strontium chloride, barium chloride, magnesium chloride, and lithium chloride. None of the solutions at pH 5.0 gelled even after 48 hours. Of those at pH 7.5, the calcium chloride showed the most marked gelling action, the strontium chloride showed somewhat less, and the barium chloride still less. The magnesium chloride and lithium chloride gave no indication of gel formation until after 7 hours, after 48 hours there were soft gels formed but even less than that formed in the control, water extract plus ammonium hydroxide to pH 7.5. The relations

[5] Colby, G. E., *California Agric. Exp. Sta. Report*, 1892–94, 275.

in the gel formations with time and concentration of salt with the first three salts were similar to those already given.

IV. The Action of Pancreatine on Banana Extract.

As a result of some experiments carried out for a different purpose, the formation of a gel by the action of pancreatine on banana extract was observed. For example, one 40 cc. portion of banana sodium chloride extract was treated at pH 5.0 with 0.2 gm. of a commercial pancreatine preparation, and another with 0.1 gm. In 24 hours at 38°, the former formed considerable gel, the latter formed less gel. At 5°, the same mixtures produced no gels. If either the pancreatine or the extract was boiled before mixing the solutions, no gel was obtained. The gel which was obtained by the action of the pancreatine appeared to be less firm than that obtained by the action of calcium salts.

A number of the gels prepared by dialysis were filtered on paper, washed with alcohol and ether and dried and analyzed. The analyses, however, mean nothing. The gels as obtained carried down mechanically or otherwise much of the dissolved material present. Attempts to wash the gels with water were not successful, swelling occurring in almost every case. Until methods of purifying the gels, or at least of removing extraneous material are developed, nothing definite can be stated relative to their chemical composition. It may be mentioned, however, that the nitrogen content of the dried gel was in the neighborhood of 4.5 per cent, pointing to the presence of about 25 per cent protein material.

DISCUSSION.

The question of the relation of this work to other work of similar nature can be disposed of briefly. The source of the material and some of the reactions may indicate that the gels are of the nature of pectins.[6] However, against this view are the facts that boiling destroys the gel-forming property and that only very small quantities of calcium and strontium salts are required to yield the gels under suitable conditions.

[6] *Cf.* Haynes, D., *Biochem. J.*, 1914, viii, 553.

The formation of the gels by the direct addition of certain salts under suitable conditions as well as by dialysis against solutions of such salts is significant. It raises the question whether it would not be possible in many cases to bring about reactions such as precipitation, solution, etc., by direct chemical actions involving the addition of reagents under definite conditions, in place of dialysis against solutions of more or less accidental salt contents. The fact that such solutions as the tap water which was used in the present investigation may contain dissolved substances in minute concentrations only serves to obscure the possible actions. These small concentrations of dissolved substances which may react, but which are constantly renewed as the dialyzing liquid changes, are capable of producing effects which larger initial concentrations would produce in shorter times. Thus, the banana gel-forming substance inside the bags was able to react with the calcium salts from the outside liquid and over the extended period of time which was used here and is also used in most dialysis studies, the reaction occurred, first along the inner walls of the bags and finally throughout the liquid. The different hydrogen ion concentrations inside and outside the bags also served to confuse the exact conditions at which the reactions could take place.

These relations make it advisable in every case in which dialysis is used to study the composition of the dialyzing liquid, as small amounts of dissolved substances and also apparently unimportant differences in hydrogen ion concentrations may result in the occurrence of definite chemical changes. Also, the material of which the dialyzing bags are made may exert profound effects upon the changes.[7] The plea may be made, therefore, to replace dialysis wherever possible by direct additions of chemical reagents at the same time controlling the hydrogen ion concentrations of the mixtures, and in this way substituting more or less accidental and to some extent unknown methods of treatment by known and definite additions of chemical reagents.

The results obtained therefore point to chemical reactions and combinations involving specific elements under definite and limited conditions. Although it was not found possible to obtain these compounds in pure condition as chemical individuals, the results can best be interpreted on the basis of the formation of such chemical compounds.

[7] Brown, W., *Biochem. J.*, 1915, ix, 591; 1917, xi, 40. Eggerth, A. H., *J. Biol. Chem.*, 1921, xlviii, 203.

SUMMARY.

The conditions for the formation of gels from banana extracts were studied.

Gels were obtained with extracts more alkaline than pH 7.0 with very small quantities of calcium, strontium, and barium salts, the gel formation with these salts decreasing in the indicated order.

In solutions more acid than pH 6.0, no gels were obtained with these salts.

Magnesium, lithium, and sodium salts did not cause gel formation either in acid or alkaline solutions.

Pancreatine gave a gel on incubation with banana extract at pH 5.0.

The gel-forming property of banana extracts was destroyed on boiling.

We wish to thank the Fruit Dispatch Company for supplying the greater part of the bananas used in this investigation.

QUANTITATIVE LAWS IN REGENERATION.

III. The Quantitative Basis of Polarity in Regeneration.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 18, 1922.)

I. INTRODUCTION.

It has been shown in preceding papers that the dry weight of shoots and roots produced under equal conditions of illumination, moisture, and temperature in sister leaves of *Bryophyllum calycinum* varies approximately in direct proportion with the dry weight of the leaves; and that the same is true for the mass of shoots produced in small pieces of stem connected with a leaf.[1] It had been known that when a piece of stem is left in connection with a leaf, the mass of shoots produced by the leaf is less than when the leaf is completely isolated, and the writer had been able to show that in this case the stem connected with the leaf gains approximately as much in dry weight as the dry weight of the shoots and roots in the leaf would have been if the leaf had been completely isolated from the stem.[2] The inhibitory influence of the stem on the shoot and root formation in the leaf was in this case due to the fact that when the leaf is connected with a stem, that part of the material which could have been utilized for the formation of new shoots and roots in the leaf now goes into the stem. It is intended to show in this paper that the same simple quantitative relations suffice to account for the polar character of regeneration in a defoliated stem of *Bryophyllum*.[3]

The reader will remember that each node of the stem of this plant has two dormant buds capable of growing into shoots. When a piece of defoliated stem is cut from a plant and suspended in moist air, only

[1] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 81; 1919–20, ii, 297, 651. *Science*, 1917, xlv, 436. *Bot. Gaz.*, 1918, xlv, 150. *Ann. Inst. Pasteur*, 1918, xxxii, 1.

[2] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 297, 651.

[3] Loeb, J., *Science*, 1921, liv, 521.

the two buds of the most apical node will grow into shoots, while the buds in all the nodes below will remain dormant. Permanent roots will grow only at the base of each piece, though transitorily air roots

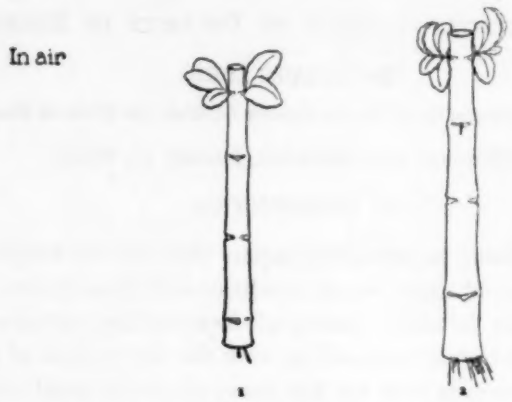

FIG. 1. Pieces of stem from the same plant, (1) apical, (2) basal. Suspended in moist air, shoots formed only in the apical node, roots at the base. Mass of shoots and roots is larger in the basal piece (2) which has the larger mass. Duration of experiment October 4 to November 7, 1921.

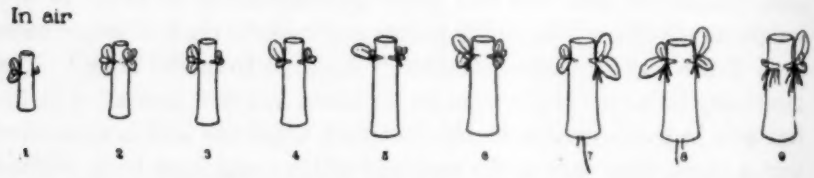

FIG. 2. Stem cut into small pieces with 1 node each. Suspended in same aquarium and simultaneously with large stems in Fig. 1. (1) was the most apical, (9) the most basal piece, the serial number denoting the original position of the pieces in the plant. Each piece of stem forms 2 shoots in its node, but the relative mass of the shoots varies with the relative mass of the stem, not with the serial number of the node.

may begin to form in any node, but these will dry out as soon as the basal roots are growing.[4] Fig. 1 illustrates this polar character of regeneration in defoliated pieces of stem suspended in moist air. When, however, a long defoliated stem is cut into as many

[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 687.

pieces as there are nodes, then all the dormant shoot buds of the stem will grow out into shoots (Fig. 2). The stems in Figs. 1 and 2 were cut out at the same time and suspended in moist air in the same vessel.

The results remain about the same when the basal ends of the pieces are dipped into water, the only difference being that often not only the

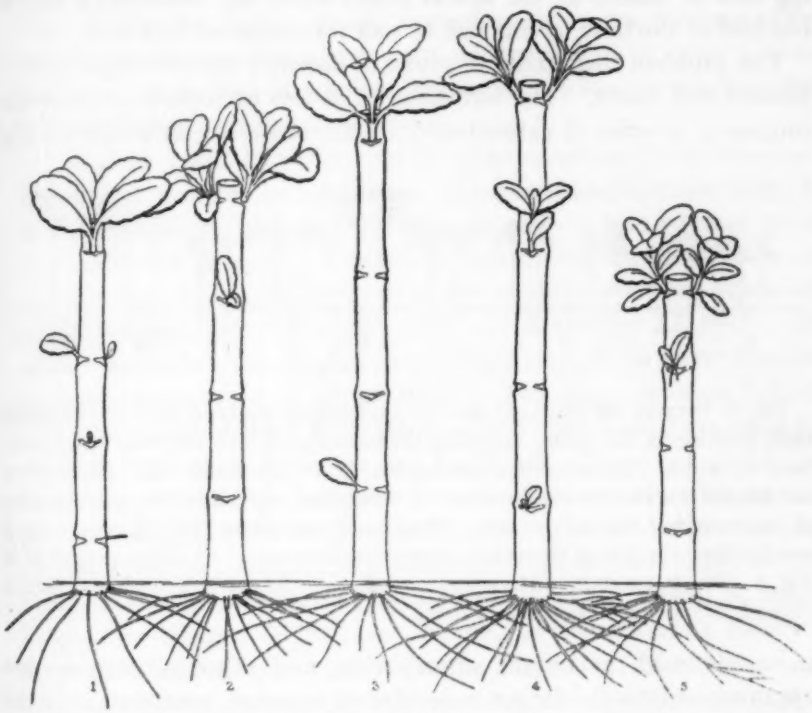

FIG. 3. Same experiments as Fig. 1, only that the long pieces of stem were put with their bases into water. Duration of experiment from September 27 to October 22, 1921. All stems were cut from one plant.

two buds in the most apical node of a long piece of stem grow out but also one or two buds of the node below (Fig. 3). The amount of growth of shoots and roots is also greater in the stems put with the base in water (Fig. 3) than when the stems are suspended in moist air (Fig. 1). When pieces of stem with only one node each are put into water, each piece forms shoots at its node (Fig. 4). The

question is, Why do only the most apical buds of a long defoliated stem grow out? Bonnet had suggested that the ascending sap of a plant was shoot-producing and the descending sap was root-producing. Sachs pointed out that when a piece of stem was cut out from a plant the ascending sap was blocked at the apex and that hence the shoot-producing substances must collect at that end of the stem giving rise to shoots at the apical node; while the descending sap is blocked at the base, giving rise to root formation at that end.

The problem then exists to prove or disprove the old suggestion of Bonnet and Sachs. The formation of shoots or roots is a synthetic process or a series of catenated synthetic processes, in which soluble

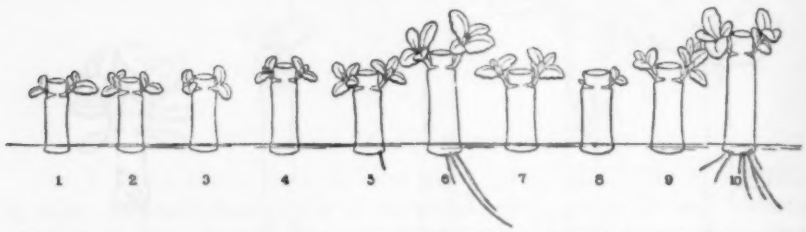

FIG. 4. Stem of one plant cut into ten small pieces, the serial number indicating their position in the plant, (1) being the most apical, (10), the most basal piece. Base in water. Experiment simultaneous with Experiment III. Each piece has formed 2 shoots the relative size of which does not follow the serial number of the stem, but the relative size. The size of each shoot of the pieces is much smaller than the size of the shoots formed simultaneously by the larger stems in Fig. 3. The latter stems all have roots, while only the two largest pieces of stems (6) and (10) in Fig. 4 have formed roots.

materials, such as sugars, amino-acids, and other substances, are synthetized into the larger molecules of proteins, compounds of the cellulose type, and others. If the theory of Bonnet and Sachs is correct, it must be possible to show that the two shoots formed at the apex of a long defoliated piece of stem have, within the limits of the accuracy of the experiments, approximately the same dry weight as the dry weight of all the shoots would have amounted to if the stem had been cut into as many small pieces as it contained nodes.

By comparing the amount of shoots formed in the one-node pieces in Fig. 2 or 4 with those of the four-node pieces in Fig. 1 or 3, the reader will notice that the shoots are greater in the larger pieces of

stem, and the same fact is obvious from all the other figures in this paper. It is almost obvious from a glance at the figures that the mass of shoots formed increases with the mass of the stem. If the mass of shoots produced at the apex of large pieces of stem is approximately equal to the mass of shoots which the same stems would have produced had they been cut into as many pieces as the stems contained nodes (*i.e.*, into one-node pieces), it will be necessary to show that within the limits of the experimental errors, the mass of dry weight of shoots produced per gram of dry weight of stem is about the same regardless of whether the stems are long or whether they are subdivided into one-node pieces.

This was tested in various ways. The defoliated stem of a very large plant was cut into 5 pieces, each possessing 4 nodes (Fig. 3), and the defoliated stem of a second plant was cut into 10 small pieces of 1 node each (Fig. 4). The pieces dipped with the base into water and the large and small pieces were suspended in the same aquarium. The experiment lasted from September 27 to October 22, 1922. The shoots were then cut off and both shoots and stems were dried for 24 hours in an oven at about 100°C. The result was as follows: The dry weight of the 5 large stems (Fig. 3) was 13.670 gm., and the dry weight of their 16 shoots was 0.495 gm. The shoot production was therefore 36 mg. per gram of stem (all measured in dry weight). The dry weight of the 10 short pieces of stem with 1 node each (Fig. 4) was 2.880 gm., and the dry weight of 19 shoots was 0.115 gm., or 1 gm. of dry weight of stem produced 40 mg. of dry weight of leaves. These two figures, 40 mg. and 36 mg., agree sufficiently closely to show that under equal conditions the production of shoots of defoliated pieces of stem occurs in proportion with the mass of the piece of (defoliated) stem; or, in other words, the mass of shoots produced at the apex of the large defoliated stems of Fig. 3 is approximately equal to the mass of shoots the same stems would have produced if all the dormant buds of each stem had been able to grow out.

The experiment in Figs. 1 and 2 gave a similar result. The experiment lasted from October 4 to November 7. 5 large stems with 4 nodes each (Fig. 1) having a dry weight of 5.486 gm. produced 10 shoots with a dry weight of 0.114 gm.; *i.e.*, 20.8 mg. of shoot per gram of stem.

4 short pieces of stem with 2 nodes each, having a dry weight of 3.214 gm., produced 8 shoots with a dry weight of 0.0668 gm.; *i.e.*, 20.7 mg. of shoot per gram of stem.

A third stem was cut into 9 pieces with 1 node each (Fig. 2) possessing a dry weight of 3.270 gm., giving rise to 17 shoots with a dry weight of 0.050 gm.; *i.e.*, 15.3 mg. of shoot per gram of stem.

The first two figures are identical, the last figure is a little low. In these experiments the end of the piece may suffer (by drying out or falling a prey to fungi) and this creates an error which is especially noticeable when a stem is cut into many small pieces. But in spite of these sources of error the results are remarkably clear and consistent.

It seemed of interest to compare the behavior of defoliated stems split longitudinally. In this case the two halves should give approximately equal results.

II. Experiments with Split Stems.

Experiments were made with stems split longitudinally as indicated in Fig. 5. Only pieces from the middle of the stem of a large plant were used, for reasons to be given later. Stems with 4 nodes each, were split longitudinally and one half was cut transversely into 2 pieces with 2 nodes each, a', b', and c', d', respectively (see Fig. 5). The other half with the 4 nodes a, b, c, and d was not cut transversely. All 3 pieces (Fig. 5) were put with their bases into water. It was to be expected that the sum of the dry weight of the shoots produced by the 2 small pieces with 2 nodes each should equal the dry weight of the shoots produced by the larger pieces with 4 nodes each. Fig. 5 shows at a glance that this is approximately the case and the dry weight determinations confirm this.

The first experiment was carried out on 7 stems, a second experiment on 16 stems. Table I gives the result.

It is therefore obvious that the dry weight of the sum of the shoots produced by the small pieces a', b', and c', d', approximately equals the dry weight of the shoots produced by the big pieces, a, b, c, and d (Fig. 5), or, in other words, the mass of shoot produced at the apex of the large pieces is approximately equal to the dry weight of the shoots the same stems would have produced if the buds of every second node had been able to grow.

TABLE I.

Experiment No.	Duration of experiment.	Number of pieces.	Dry weight of shoots produced.	Dry weight of stems.	Shoots produced per gram of stem.
	1921		*gm.*	*gm.*	*mg.*
I	Nov. 3–Dec. 6.	7 four-node pieces, *a*, *b*, *c*, *d*.	0.1545	4.290	36.0
		14 small pieces, *a'*, *b'*, *c'*, *d'*.	0.147	3.822	38.7
	1921-22				
II	Dec. 8–Jan. 10.	16 four-node pieces, *a*, *b*, *c*, *d*.	0.750	16.646	45.0
		32 two-node pieces, *a'*, *b'*, *c'*, *d'*.	0.577	14.527	39.5

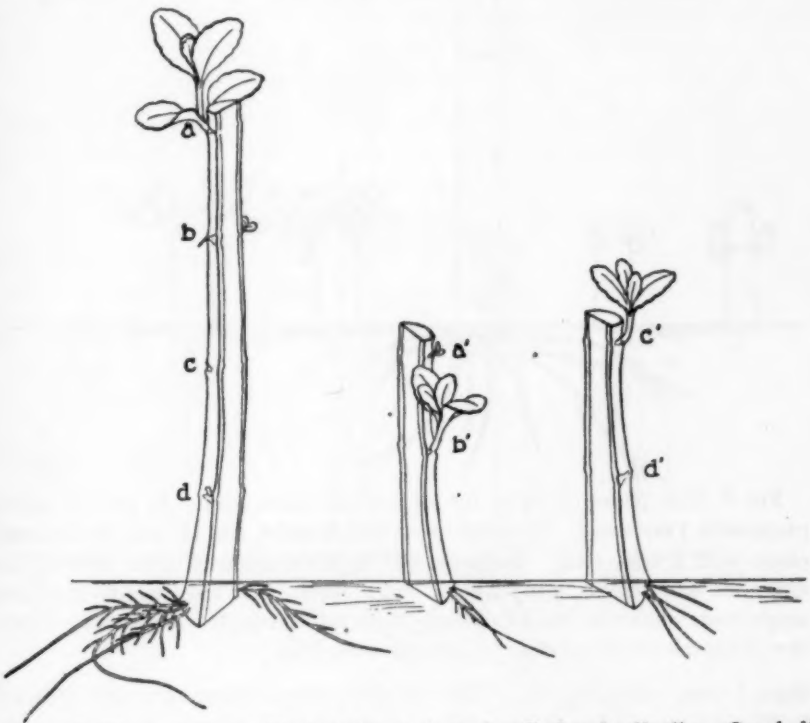

FIG. 5. Piece of stem with 4 nodes, *a*, *b*, *c*, *d*, split longitudinally. One half cut transversely into two pieces, *a'*, *b'*, and *c'*, *d'*. The half *a*, *b*, *c*, *d*, produces 1 shoot which about equals in mass the 2 shoots produced by *a'*, *b'*, and *c'*, *d'*. Duration of experiment December 9, 1921, to January 4, 1922.

III. Small and Large Pieces of the Same Stem.

A third series of experiments was as follows: Long pieces of stem, containing about 10 nodes, were cut out from the same plant more

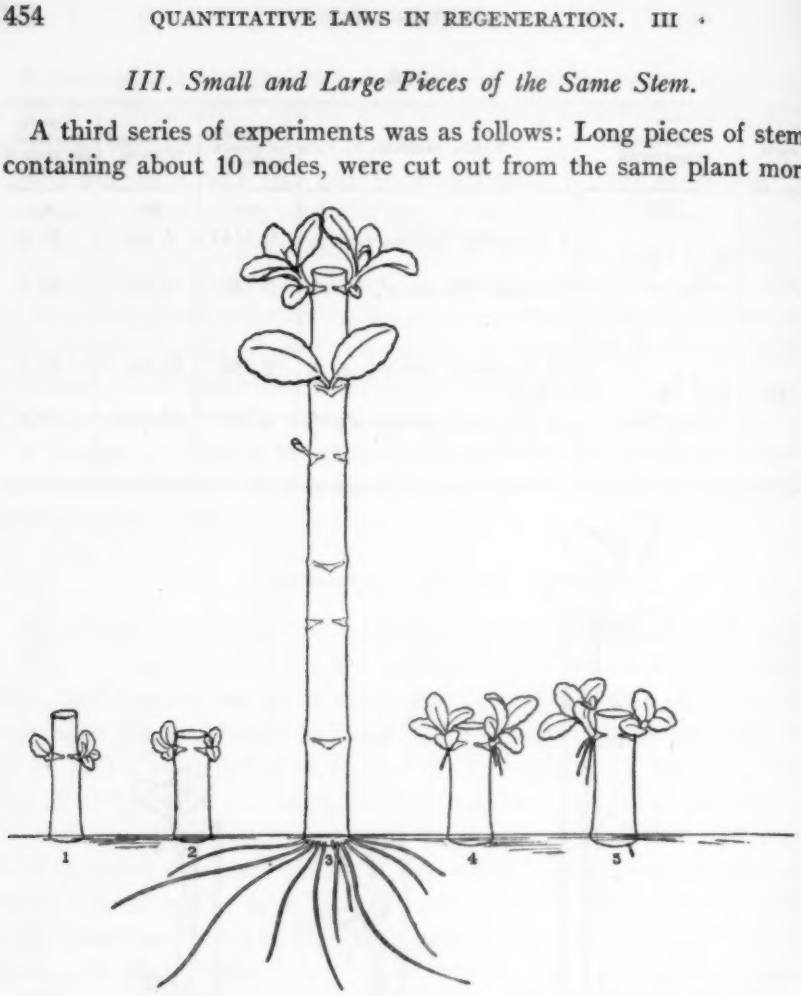

FIG. 6. Five pieces cut from the stem of the same plant, (1) and (2) apical pieces with 1 node each, (3) middle piece with 6 nodes, and (4) and (5) the basal pieces with 1 node each. The large middle piece produces larger shoots than either the more apical or more basal small pieces. The large middle piece has ample roots while only the longer basal piece commences to form a root. Duration of experiment October 25 to November 21, 1921.

than 1 year old (Fig. 6). The middle piece of about 6 nodes (piece 3 in Fig. 6) served for the experiment, two small pieces, 1 and 2, containing 1 node each and situated apically, and 2 small pieces, 4 and 5, also containing 1 node each, situated basally from the large middle

piece in the same stem, serving as controls. In other experiments of the same character, pieces containing about 14 nodes were cut out from the stem of the same plant; 2 small pieces at the apex, each containing 2 nodes (1 and 2, Fig. 7), and 2 small pieces at the base each

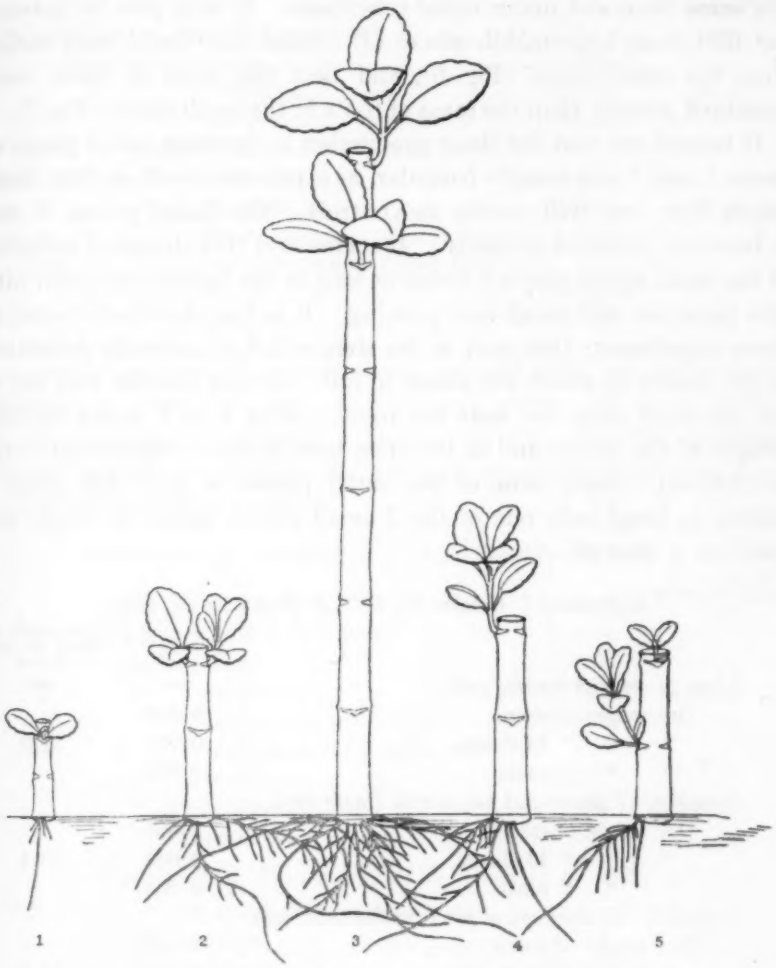

FIG. 7. Similar experiment as Fig. 6, except that the small pieces have 2 nodes each. Shoots and roots are formed in proportion to mass of stem. Duration of experiment November 16 to December 19, 1921.

containing also 2 nodes (4 and 5, Fig. 7), were used as controls, while the middle piece (3, Fig. 7) served for the main experiment. All the pieces dipped with their bases into water.

It is obvious from Fig. 6 that the large pieces of stem (3) produced larger masses of shoots than the small pieces 1 and 2 or 4 and 5 during the same time and under equal conditions. It may also be pointed out that these large middle pieces (3) formed their basal roots earlier than the small pieces (Fig. 6), and that the mass of their roots remained greater than the mass of roots in the small pieces (Fig. 7).

It turned out that the shoot production in the most apical pieces of stems 1 and 2 was usually irregular, as a rule too small, so that these pieces were not well usable as controls. The basal pieces, 4 and 5, however, behaved normally. It seems that this abnormal behavior of the small apical pieces is found as long as the leaves connected with this piece are still small and growing. It is therefore well to use in these experiments that part of the stem which is naturally defoliated or the leaves of which are about to fall. It may also be well not to use pieces of stem too near the roots. After 3 to 5 weeks the dry weight of the shoots and of the stem used in these experiments were determined. Since some of the small pieces of stem fall often a victim to fungi only one of the 2 small pieces, apical or basal, was used as a control.

Experiment I. October 25, 1921, to November 25, 1921.

	gm.	Dry weight of shoots per gram of stem. mg.
6 long pieces with 6 nodes each.		
Dry weight of stems	9.260	
" " " 13 shoots	0.260	28.0
" " " roots	0.057	
Control a. 7 short basal pieces with 1 node each.		
Dry weight of stems	2.895	
" " " 13 shoots	0.088	30.4
" " " roots	0.003	
Control b. 12 short apical pieces with 1 node each.		
Dry weight of stems	1.428	
" " " 18 shoots	0.0236	16.5

It is obvious that the apical control pieces gave too small a production of shoots (16.5 mg. per gram of stem), while the basal con-

trol pieces produced approximately the same amount of shoots per gram of stem, namely 30.4 mg. as compared with 28.0 for the large pieces.

Experiment II. November 2, 1921, to December 6, 1921.

	gm.	Dry weight of shoots per gram of stem. mg.
5 long pieces of stem with 6 nodes each.		
Dry weight of stems	6.486	
" " " 10 shoots	0.272	42.0
" " " roots	0.0458	
Control a. 4 short basal pieces with 1 node each.		
Dry weight of stems	1.058	
" " " 8 shoots	0.041	39.0
" " " roots	0.0034	
Control b. 5 short apical pieces with 1 node each.		
Dry weight of stems	0.544	
" " " 10 shoots	0.018	33.0

Again the short basal control pieces produce about as much shoot material per gram (39 mg.), as the large pieces (42 mg.), while the apical controls produce less, namely, 33 mg. We will omit the apical controls in the further tabulation of experiments on account of the irregularity of the results.

Experiment III. November 16, 1921, to December 20, 1921.

	gm.	Dry weight of shoots per gram of stem. mg.
9 long pieces of stem with 6 nodes each.		
Dry weight of stems	18.658	
" " " 26 shoots	0.944	50.3
" " " roots	0.1428	
Control. 18 small basal pieces of 2 nodes each.		
Dry weight of stems	18.147	
" " " 36 shoots	0.800	44.0
" " " roots	0.136	

Experiment IV. October 22, 1921, to November 15, 1921.

4 long pieces of stem with 4 nodes each.		
Dry weight of stems	4.214	21.0
" " " 8 shoots	0.089	
Control. 4 short basal pieces of 2 nodes each.		
Dry weight of stems	2.492	19.0
" " " 8 shoots	0.0475	

Experiment V. October 11, 1921, to November 1, 1921.

	gm.	Dry weight of shoots per gram of stem. mg.
4 long apical stems with 6 nodes each.		
Dry weight of stems	3.921	
" " " 8 shoots	0.113	29.0
" " " roots	0.0134	
Control. 4 basal pieces of 2 nodes each.		
Dry weight of stems	3.744	24.0
" " " 10 shoots	0.090	

Experiment VI. December 11, 1921, to January 17, 1922.

7 long apical stems with 6 nodes each.		
Dry weight of stems	6.634	
" " " 12 shoots	0.340	51.0
" " " roots	0.0512	
Control. 7 short basal pieces of 2 nodes each.		
Dry weight of stems	3.560	
" " " 12 shoots	0.1770	49.6
" " " roots	0.0128	

If we consider only those figures in the experiments where the small control pieces of stem were situated basally from the long stem (the pieces 4 and 5 in Figs. 6 and 7), we notice that the differences of shoots produced per gram of dry weight of the controls differ never more than 25 per cent from those produced by the large pieces of stem and that in some cases the difference is only about 6 per cent. Considering the limitations in the experimental conditions—especially the fact that part of the stem may not function normally, especially the ends near the cut, or the fact that individual buds may have been injured by parasites, etc.—the agreement of the figures seems remarkable.

These results leave no doubt that within the limits of accuracy of these experiments the dry weight of the shoots produced at the apex of a long piece of defoliated stem is about equal the mass of shoots the same stem would have produced had the buds in all of its nodes been able to develop.

IV. Regeneration of Roots.

Two kinds of roots are formed in an isolated piece of stem, suspended in moist air and dipping with the base in water, first, air roots in the nodes, and later roots at the basal end of the stem regardless of the node (Figs. 1, 3, and 7). The air roots in the nodes grow out sooner than the basal roots but as soon as the basal roots grow out the air roots dry out and die. This has been discussed in a previous paper. We are interested here only in the basal roots since they alone are connected with the problem of polarity. The regeneration of the basal roots differs from the formation of apical shoots in this, that the apical shoots begin to grow out almost immediately after the defoliated piece of stem is isolated, while there is a long latent period before the basal roots make their appearance. For this reason quantitative measurements correlating the mass of the basal root formation with the mass of stem require probably a longer time than that in our experiments. A glance at the drawings will, however, convince the reader that the root formation commences sooner in the stems with larger mass than in the stems with smaller mass, regardless of the original position of the piece of stem in the plant.

Thus in Fig. 6 the large middle piece (3) forms roots before either of the 2 more basal pieces form roots, and Fig. 7 shows that the relative mass of roots produced seems also to run parallel with the relative mass of the piece. The same phenomenon is shown if we compare Fig. 1 with Fig. 2, or Fig. 3 with Fig. 4. It is also obvious in Fig. 5, so that we can say that the mass of roots produced by pieces of defoliated stem of *Bryophyllum calycinum* increases under equal conditions with the mass of the stem.

V. Influence of Light on Regeneration in a Defoliated Stem.

8 long defoliated stems were suspended into an aquarium kept dark by a double cover of black cardboard, and 8 equally long defoliated stems were put at the same time into an aquarium exposed in the usual way to daylight. The base of the stems dipped into water. All conditions were equal except the illumination. After 23 days all the stems exposed to light had formed large basal roots and large shoots at the apex (Fig. 8). At the same time none of the stems

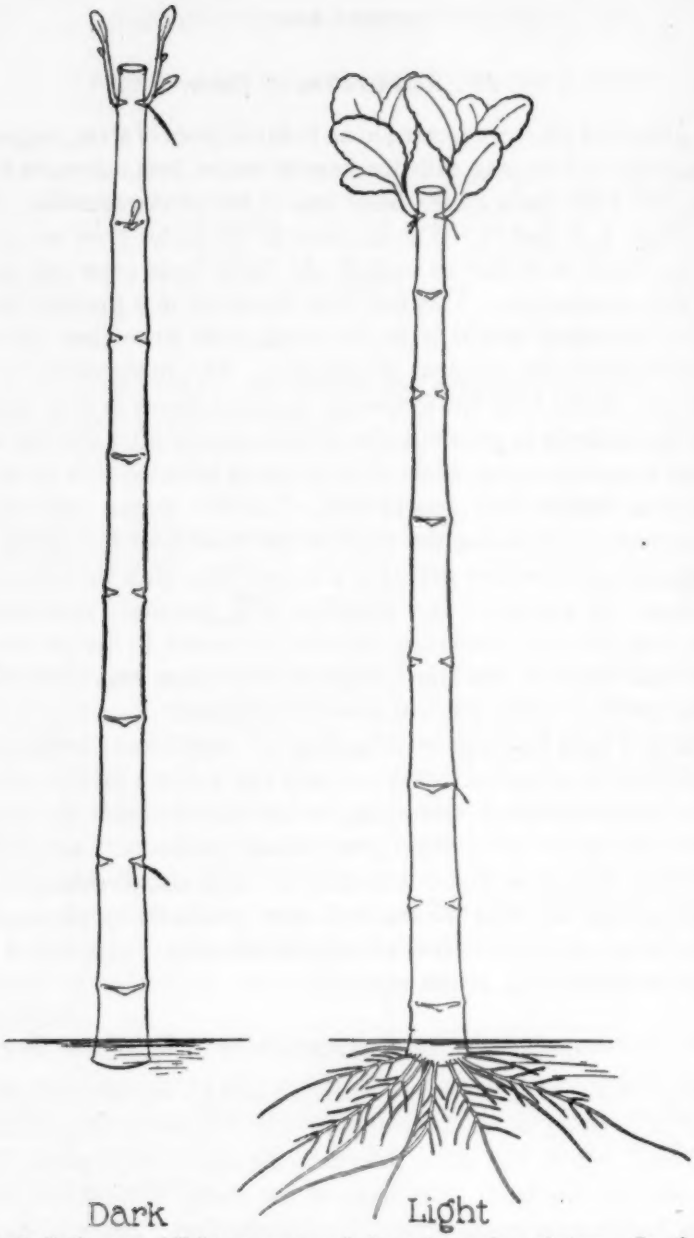

FIG. 8. Influence of light on root and shoot formation of stem. In the dark no roots are formed; in light ample roots are formed. The mass of shoots formed in dark is small compared with mass of shoots formed in light.

in the dark had formed a single basal root though some had formed tiny air roots (Fig. 8). The shoots formed in the dark had a small mass and the typical etiolated shape. The most striking phenomenon was the lack of root formation at the base of the stem in the dark. The writer had already shown that the favorable influence of the leaf on root formation in the stem also disappears when the leaf is deprived of light.[4]

SUMMARY AND CONCLUSION.

It is well known that a long defoliated piece of stem of *Bryophyllum calycinum* forms shoots only at the apical or the two apical nodes, while when such a stem is cut into as many pieces as there are nodes each node produces shoots. It is shown in this paper that the dry weight of shoots produced in the apical nodes of a long piece of stem is approximately equal to the dry weight of shoots the same stem would have produced if it had been cut into as many pieces as it possesses nodes. Hence all the material which can be used for the growth of shoots goes into the most apical part of the stem and this accounts for the polar character of regeneration in this case.[5]

It seems that the mass of basal roots produced by a piece of defoliated stem also increases with the mass of the stem.

[5] A plant morphologist, to whom the writer showed these experiments, commented that he was convinced that the shoot formation of an isolated piece of stem was due to a "stimulus." If we accept this suggestion, it follows that the "stimulus" for regeneration must have varied quantitatively with the mass of the defoliated stem in our experiments, and this would lead us again to the idea that the "stimulus" must be something material since it cannot well be spiritual.

ELECTRICAL CHARGES OF COLLOIDAL PARTICLES AND ANOMALOUS OSMOSIS.

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I. The Transport Curves on the Acid Side of the Isoelectric Point of the Membrane.

The experiments described in a preceding paper[1] leave no doubt that the Donnan equilibrium is the main source of the potential differences between solid gelatin particles and the surrounding liquid. On the other hand, experiments on the influence of salts on electrical endosmose, cataphoresis, anomalous osmosis, and Quincke's current potentials suggest in certain cases at least a second source which is generally designated as adsorption potentials. The difference between the two kinds of potentials should be that while the potential differences due to the Donnan equilibrium depend on the ionization of the protein, the adsorption potentials should occur regardless of whether or not the solid colloid is ionized. Adsorption potentials should, therefore, be found just as well in the case of isoelectric protein where the protein is practically non-ionized as in the case of metal proteinates or protein-acid salts, while the P.D. due to the Donnan equilibrium should be restricted to the latter two forms of protein.[2]

It is intended to investigate on the basis of this idea whether or not there exist at the surface of solid gelatin adsorption potentials in addition to potentials due to the Donnan equilibrium. It will be necessary to use for this purpose either electrical endosmose or anomalous osmosis or Quincke's current potentials or cataphoresis. We shall select in this paper anomalous osmosis. By anomalous osmosis

[1] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 351.

[2] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 667; 1921–22, iv, 351.

is meant the superposition of electrical forces over the purely osmotic forces in the transport of water through a membrane separating pure water from a solution of an electrolyte (or separating two different solutions of electrolytes). When both water and electrolytes are capable of diffusing through the membrane the difference in the mobility of the oppositely charged ions will cause diffusion potentials acting across the membrane. In this case the solution assumes the opposite sign of charge as the water. These potentials we will call E.

There may be a second P.D. inside the pores of the membrane between the solid wall of the pore and the liquid inside the pore. This potential we will call ϵ. If as the consequence of ϵ the liquid inside the pore assumes a negative charge, while as a consequence of E the solution in the collodion bag assumes a positive charge, the liquid cylinder inside the pore will be dragged into the solution by these electrical forces and thus an electrical transport of water will be added to the transport of water by osmotic forces. If the solution, however, has the same sign of charge as the liquid inside the pore, the electrical force will act in an opposite sense from the osmotic force, and the flow of water from the water side of the membrane into the solution will be less than is to be expected on the basis of van't Hoff's law.

This theory of anomalous osmosis was first suggested by Girard[3] and has later been supported by Bartell[4] and others.

In the experiments to be described salt solutions of a definite pH but of different concentrations were put into collodion flasks of about 50 cc. volume, which had received a coating of gelatin as described in previous papers. The collodion bags were dipped into 350 cc. of water of the same pH as that of the salt solution, but containing no salt. The collodion bags were closed with a rubber stopper perforated by a glass tube serving as manometer. The temperature was 24°C. and the rise of the liquid in the manometer was read 20 minutes after the commencement of each experiment. In the close regulation of

[3] Girard, P., *Compt. rend. Acad.*, 1908, cxlvi, 927; 1909, cxlviii, 1047, 1186; 1910, cl, 1446; 1911, cliii, 401. Girard, P., La pression osmotique et le méchanisme de l'osmose, Publications de la Société de Chimie-physique, Paris, 1912.

[4] Bartell, F. E., *J. Am. Chem. Soc.*, 1914, xxxvi, 646. Bartell, F. E., and Hocker, C. D., *J. Am. Chem. Soc.*, 1916, xxxviii, 1029, 1036. Bartell, F. E., and Madison, O. E., *J. Physical Chem.*, 1920, xxiv, 593.

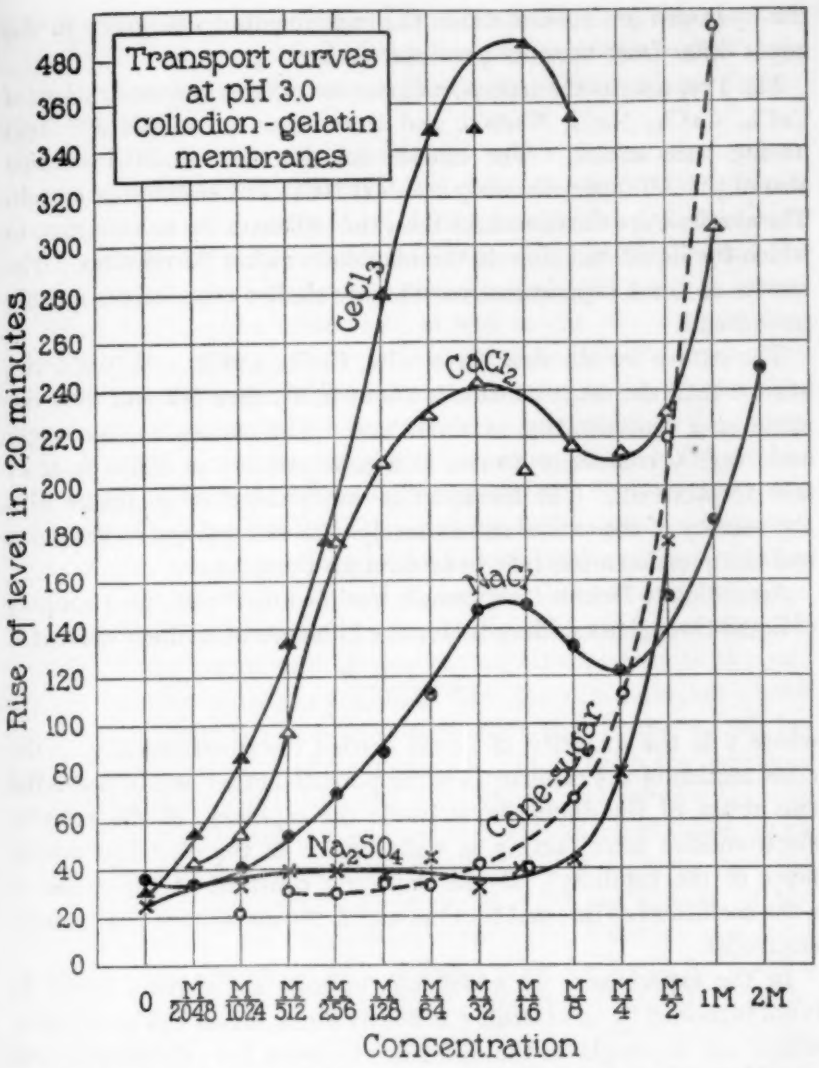

FIG. 1. Transport curves of liquid from the side of water to the side of salt solution through collodion-gelatin membranes of pH 3.0. Abscissæ are the concentration of salt, ordinates the rise in mm. of level in manometer in solution after 20 minutes. Notice the difference between the transport curve for cane-sugar and those for NaCl, $CaCl_2$, and $CeCl_3$.

the hydrogen ion concentration the experiments to be given in this paper differ from those of previous workers.

Fig. 1 represents the transport curves for different concentrations of $CeCl_3$, $CaCl_2$, NaCl, Na_2SO_4, and cane-sugar, all of pH 3.0 (HCl having been added). The outside solution was an HCl solution also of pH 3.0 (approximately N/1,000 HCl), but containing no salt. The abscissæ are the concentrations, the ordinates the level in mm. to which the liquid had risen in the manometer after 20 minutes. The results of these experiments corroborate similar experiments already published.[5]

The curves for the first three salts, $CeCl_3$, $CaCl_2$, and NaCl, rise at first until the concentration is about M/32, then fall and then rise again at a concentration of about M/4. The curves for cane-sugar and Na_2SO_4 commence to rise at a concentration of about M/32 or M/8 respectively. The transport of water therefore increases with the valency of the cation and inversely with the valency of the anion and the question arises how to account for these curves.

According to Helmholtz's formula modified by Perrin, the transport of liquid through a capillary under the influence of a direct current is

$$v = \frac{q \cdot \epsilon \cdot E \cdot D}{4\pi \cdot \eta \cdot L}$$

where v is the quantity of liquid carried electroosmotically, q the cross section of the capillary, ϵ is the potential difference between the two strata of the double layer inside the capillary, E the external electromotive force (acting at right angles to the electrical double layer in the capillary), D the dielectric constant of the medium, η the coefficient of internal friction, and L the distance of the external electrodes.

In the experiments on anomalous osmosis the driving force, E, is not furnished by an external P.D. but by a P.D. across the membrane, which has its origin in the difference between the solutions on the opposite sides of the membrane and which acts only through the extremely small distance of the thickness of the membrane. ϵ is the P.D. between the liquid inside the pores and the wall of the gelatin film. At pH 3.0, gelatin exists in the form of gelatin-acid salts, *e.g.* gelatin

[5] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 387, 563, 577, 659, 673.

chloride, when the acid is HCl. The water inside the pores of a film of gelatin chloride is negatively charged while the gelatin is positively charged. This is a consequence of the theory of Donnan's membrane equilibrium.[1]

In these experiments the gelatin with which the membrane was coated was originally isoelectric, but the film of gelatin was brought into equilibrium with water of pH 3.0 by putting the collodion bags for several hours into HCl of pH 3.0 before the beginning of the experiment. Since the addition of salts influences both the P.D. across the membrane (*i.e.* the value of E) as well as the P.D. between the liquid inside the pores and the wall of the pore (*i.e.* the value of ϵ) it is necessary to measure the influence of salts on these two P.D. separately and then try to use the results for the analysis of the curves in Fig. 1. The measurements of the P.D. across the membrane were made with the aid of a Compton electrometer and the two electrodes used were calomel electrodes with saturated KCl solution. Measurements of the P.D. across the membrane were made at the beginning of the experiment (Table I) and at the end; *i.e.*, after 20 minutes (Table II). The figures for the P.D. in Table II are lower than in Table I for the reason that during the experiment part of the salt diffused into the outside solution so that the concentration of the salt solution inside the bag diminished while that in the outside increased; hence the value of E diminished. In addition the hydrogen ion concentration which was the same inside and outside the salt solution at the beginning changed and this added a complication which has been discussed in a previous paper.[6] Table I shows that the P.D. across the membrane (E) increases with the valency of the cation and inversely with the valency of the anion, E being a maximum for $CeCl_3$, being lower for $CaCl_2$, and still lower for NaCl; for Na_2SO_4 it becomes about zero or even slightly negative (Table II). It is, therefore, obvious that in a semiquantitative way the results of Tables I and II (*i.e.* the values of E) explain the difference in the ascending branches of the curves in Fig. 1 up to a concentration of about M/32.

It can be shown that this P.D. is at least partly due to diffusion potentials. In measuring the diffusion potentials the principle of a

[6] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 213.

TABLE I.

Influence of Concentration of Salt on the Value of E.

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 3.0 against H_2O of pH 3.0 (acid used, HCl), *at beginning of experiment.* Sign of charge of solution always positive unless minus sign is added.

Concentration.	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
	millivolts	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
$CeCl_3$.....	1.0	9.0	15.0	21.0	32.0	41.0	45.0	41.0	41.0	41.5				
$CaCl_2$.....	0.0	8.0	16.0	16.0	23.0	25.0	35.0	34.0	33.0	39.0	42.5	47.0	49.0	
NaCl.....	1.5	5.5	8.0	12.0	13.0	18.0	22.0	20.0	23.0	23.0	24.0	25.0	28.0	28.0
Na_2SO_4.....	0.5	5.0	6.0	5.0	8.5	7.5	5.0	−1.5	−2.0	−8.0	−12.0	−17.5		
Cane-sugar.....			2.0	2.0	0	0	0	−1.0	0	0	− 0.5	1.0	0	

TABLE II.

Influence of Concentration of Salt on the Value of E.

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 3.0 against H_2O of pH 3.0 (acid used, HCl), *after 20 minutes* from beginning of experiment. Sign of charge of solution always positive unless minus sign is added.

Concentration.	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
	millivolts	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
$CeCl_3$.....	−0.5	10.0	16.0	22.5	28.0	35.0	41.0	33.0	32.0	29.0				
$CaCl_2$.....	−1.0	6.5	11.5	17.0	22.0	23.0	30.0	27.5	25.0	25.0	24.0	23.0	20.0	
NaCl.....	1.5	4.0	7.5	12.0	10.0	13.0	16.5	16.0	16.0	15.0	11.0	12.0	10.5	8.0
Na_2SO_4.....	0	3.5	1.0	1.0	1.0	−0.5	−2.5	−6.0	−6.0	−10.0	−10.0	−13.0		
Cane-sugar.....			3.0	6.0	2.0	2.0	3.0	4.0	0	1.0	3.0	5.0	1.0	

flowing junction of Lamb and Larson[7] was used in a simplified and also less accurate form, which, however, gave results of sufficient accuracy for our purpose. Table III gives some of the results showing that the order of efficiency of the various salts and the influence of concentration are the same in diffusion potentials as in the P.D. across the membrane. The diffusion potentials are, however, considerably lower than the P.D. across the membrane (Tables I and II). The diffusion potentials depend on the difference in the relative velocity of the oppositely charged ions of a salt. A comparison of the values in Table I and Table III suggests that the source of the P.D. across the membrane is the same as that of the diffusion potential if we assume that at pH 3.0 the cations experience a greater retardation in the diffusion through protein films than anions.

The figures in Table I, II, or III do, however, not explain the drop in the curves of Fig. 1 which occurs when the concentration of the salt reaches M/32. The cause for this depression lies probably in the influence of the concentration of the salt on the value of ϵ; *i.e.*, P.D. between gelatin chloride and water inside the pores of the membrane. It has been shown in previous experiments that salts depress the P.D. between gelatin particles and surrounding liquid and that the reason for this depression is furnished by the Donnan theory of membrane equilibria.[1] The method of these experiments was briefly as follows:

1 gm. of fine particles of powdered gelatin rendered first isoelectric and of an equal size of grain was put into 200 cc. of various concentrations of a salt (NaCl, $CaCl_2$, etc.) in water and containing 8 cc. of 0.1 N HCl per 100 cc. The gelatin remained in this solution for 2 hours at 20°C. under frequent stirring. The suspension was then put on a filter and the gelatin freed from the supernatant liquid. The gelatin was then melted by heating to 45° and cooled rapidly to cause solidification and the P.D. between the gelatin and the supernatant liquid was then measured with the aid of a Compton electrometer. Table IV gives the result. The reader will notice that in no case do any of the salts cause a rise in the P.D. between gelatin and liquid. The observed P.D. could, however, be calculated with a fair degree of accuracy from Donnan's equilibrium equation.

[7] Lamb, A. B., and Larson, A. T., *J. Am. Chem. Soc.*, 1920, xlii, 229.

TABLE III.

Diffusion potentials of different concentrations of salts of pH 3.0 against H_2O of pH 3.0 (acid used, HCl). Sign of charge of salt solution always positive unless minus sign is added.

Concentration.	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M
	millivolts	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
$CeCl_3$		2.0	4.0	7.0	10.0	16.0	19.0	24.0	31.0	36.0			
$CaCl_2$		2.0	3.0	6.0	10.0	13.0	17.0	22.0	26.0	30.0	35.0	43.0	47.0
NaCl	0	0	0.5	1.5	3.0	5.0	7.0	9.0	12.0	15.0	15.0	17.0	18.0
Na_2SO_4		−1.0	−1.0	−1.5	−2.0	−3.0	−5.0	−7.0	−9.0	−12.0	−15.0	−22.0	
Cane-sugar		0	0	0	0	0	−0.5	−0.5	−0.5	− 1.0	− 1.5	− 3.0	−3.0

TABLE IV.

Influence of Different Concentrations of Salts on the Value of ϵ.

P.D. in millivolts between solid gelatin particles and HCl solution. pH of gelatin particles about 2.8 (in absence of salt).

Concentration.	0	M/8192	M/4096	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8
	millivolts	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
NaCl	25.5	23.0	25.0	25.0	23.0	21.5	18.5	14.0	10.5	7.0	5.5	2.5
$CaCl_2$	25.5	25.5	23.0	24.0	21.0	19.0	15.5	11.5	7.5	5.0	3.0	2.5
$BaCl_2$	26.0	25.0	24.0	23.0	22.0	18.5	15.0	11.0	7.5	5.5	2.5	2.0
$CeCl_3$	26.0	25.0	22.0	21.5	19.0	16.0	11.5	8.0	5.0	2.5	2.5	
Na_2SO_4	25.0	22.0	22.5	21.0	18.0	16.0	11.5	8.5	6.5	4.0	3.0	1.5

The effect of the salt on ϵ was depressing and no increase in ϵ is noticeable in the low concentrations of $CeCl_3$ or $CaCl_2$. It is therefore obvious that the effect of the salts on ϵ exhibited by our method of experimenting can account for the drop in the curves in Fig. 1 but apparently not for the initial rise or the augmenting effect of low concentrations of $CeCl_3$ or $CaCl_2$. If Helmholtz's formula holds for these experiments curves representing the product of the values $E \times \epsilon$ should therefore show an initial rise, followed by a drop; moreover, they should show the relative order of the rise as exhibited in the transport curves in Fig. 1.

In Fig. 2 the curves for $E \times \epsilon$ are plotted with the concentration of the salt as abscissæ and the value of $E \times \epsilon$ as ordinates. The values of ϵ are taken from Table IV and those for E from Table II. The general order of the four curves in Fig. 2 is sufficiently similar to that in Fig. 1 to indicate that our interpretation of the curves in Fig. 1 is approximately correct. Thus the transport curve for Na_2SO_4 in Fig. 1 is flat, and so is the $E \times \epsilon$ curve for Na_2SO_4 in Fig. 2. The curves for NaCl, $CaCl_2$, and $CeCl_3$ rise in both figures in the order named and in both curves the rise is followed by a drop. The second rise of the curves in Fig. 1 after a concentration of M/4 is due to the osmotic effect and has no connection with the electrical effect, as is shown by the fact that this rise occurs also in the cane-sugar curve. Hence, as far as the electrical effect in Fig. 1 is concerned, the character of the curves resembles that of the curves in Fig. 2, as is proven by the fact that if we superpose the curves for $E \times \epsilon$ in Fig. 2 over the transport curve for cane-sugar in Fig. 1 we get the curves of the type of Fig. 1. This is still more approximately the case if we substitute for the purely osmotic transport effect not the curve for cane-sugar but the curves for NaCl and $CaCl_2$ at the isoelectric point of gelatin (Fig. 4). Since the curve for NaCl at pH 4.7 (Fig. 4) rises more slowly than the corresponding curve for $CaCl_2$, the lowest point in the NaCl curve in Fig. 1 must be lower than that for $CaCl_2$.

This explains the empirical rule at which the writer had arrived in his previous papers[5] on anomalous osmosis; namely, first, that the transport of water through the collodion-gelatin membrane from the side of the water to the side of solution is increased by that ion of the salt which has the same sign of charge as the membrane, and dimin-

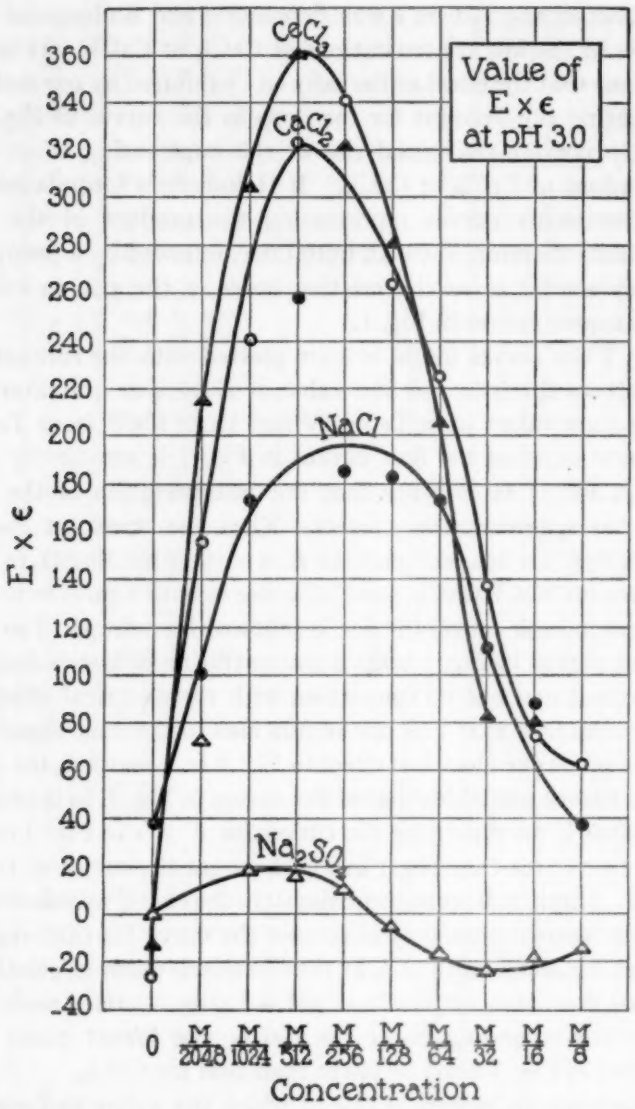

FIG. 2. Value of product of P.D. across the membrane (E) and P.D. inside the pores of the membrane (ϵ) at pH 3.0. Abscissæ are the concentration of salt solution, ordinates $E \times \epsilon$. Notice similarity of curves for $E \times \epsilon$ with the curves in Fig. 1 at lower concentrations of salt.

ished by the oppositely charged ion with a force increasing with the valency of the ion; and second, that the relative effect of the oppositely charged ions is not the same at different concentrations, but that at low concentrations the augmenting effect of the ion with the same sign of charge as the membrane increases more rapidly than the depressing effect of the oppositely charged ion, while at high concentrations the reverse is true. The augmenting effect of the ion with the same sign of charge as that of the membrane on the transport of liquid is due chiefly, if not exclusively, to the effect of the salt on the P.D. across the membrane (E), which depends upon that ion which gives the salt solution the opposite charge from that of the liquid inside the pores. This ion is in this case the cation and hence the transport increases with the valency of the cation (Tables I and II). The depressing effect of the oppositely charged ion on the transport of liquid is due to the effect on ϵ. If ϵ is determined by the Donnan equilibrium between the solid gelatin salt and the bounding solution, it must, according to the theory, be depressed by that ion which has the opposite sign of charge as the protein ion.[1] The oppositely charged ions of a salt act, therefore, each on a different type of P.D., the one on diffusion potentials, the other on a P.D. due to an equilibrium condition.

There are three discrepancies between the curves in Figs. 1 and 2 which need further explanation. First, the location of the maximum of the curves in Figs. 1 and 2 is not identical, being located at M/32 in Fig. 1 and at M/256 or M/512 in Fig. 2. This may be partly or entirely due to the fact that the concentration of the liquid was lower inside the pores than in the salt solution since water was flowing constantly from the side of water into the solution, thus causing a considerable dilution inside the pores.

Second, the curves in Fig. 1 do not come down to zero while those in Fig. 2 come down to nearly zero at a nominal concentration of M/8. For this we may have two reasons, first, that when the concentration exceeds M/4 the transport due to osmotic forces becomes so great that a drop of the transport curves to zero is no longer possible; or it may mean that after the concentration exceeds M/4 a new source of electrification of the gelatin inside the pores not accounted for by the ionization of the protein commences. We shall return to this

possibility later and show that there is no adequate support for this second assumption, though it cannot be absolutely excluded.

Third, the difference between the curves for $CeCl_3$ and $CaCl_2$ is smaller in Fig. 2 than in Fig. 1. It is possible that Ce increases the value of ϵ beyond that accounted for by the ionization of gelatin chloride.

Aside from these discrepancies we can say that Helmholtz's formula explains the curves for anomalous osmosis given in Fig. 1 when the values for ϵ used are those to be expected on the basis of the Donnan equilibrium. It may, therefore, be stated that the Donnan theory is able to explain the phenomena of anomalous osmosis more completely than any other theory thus far offered.

II. The Transport Curves on the Alkaline Side of the Isoelectric Point.

In these experiments the salt solutions were rendered alkaline by adding enough KOH to bring the salt solution to a pH of 11.0. The outside solution was a pure KOH solution also of pH 11.0 but free from salt. Fig. 3 gives the curves for the transport of liquid in the solution during the first 20 minutes. The curves show a rise—until the concentration of the salt is about M/64—followed by a drop, and then a second rise follows at a concentration of about M/8. The general character of the curves in Fig. 3 is about the same as that in Fig. 1 but the relative efficiency of the cations and anions is reversed. In solutions whose pH is on the alkaline side of the isolectric point of gelatin, the "attraction" of the solution for water increases with the valency of the anion but inversely with the valency of the cation; while on the acid side the relative efficiency of the two oppositely charged ions is the reverse. Thus in Fig. 1 the curve for Na_2SO_4 is flat while that for $CaCl_2$ rises; in Fig. 3 the curve for Na_2SO_4 rises while that for $CaCl_2$ is flat. The reason for this reversal is the fact that the sign of charge between the liquid inside the pores of the gelatin film and the gelatin wall of the pore is reversed on the opposite sides of the isoelectric point. At pH 3.0 the gelatin is positively charged and the liquid inside the pores is negatively charged; while at pH 11.0 the gelatin is negatively charged and the liquid inside the pores is positively charged. The sign of charge of the solution in the P.D. across the membrane, *i.e.* of E, remains, however, the same in alkali and acid solutions (Table V).

Table V gives the P.D. across the membrane at the beginning of the experiments represented in Fig. 3 and Table VI gives the diffusion potentials between the same salt solutions of pH 11.0 against KOH of pH 11.0 without salts and with no membrane between them.

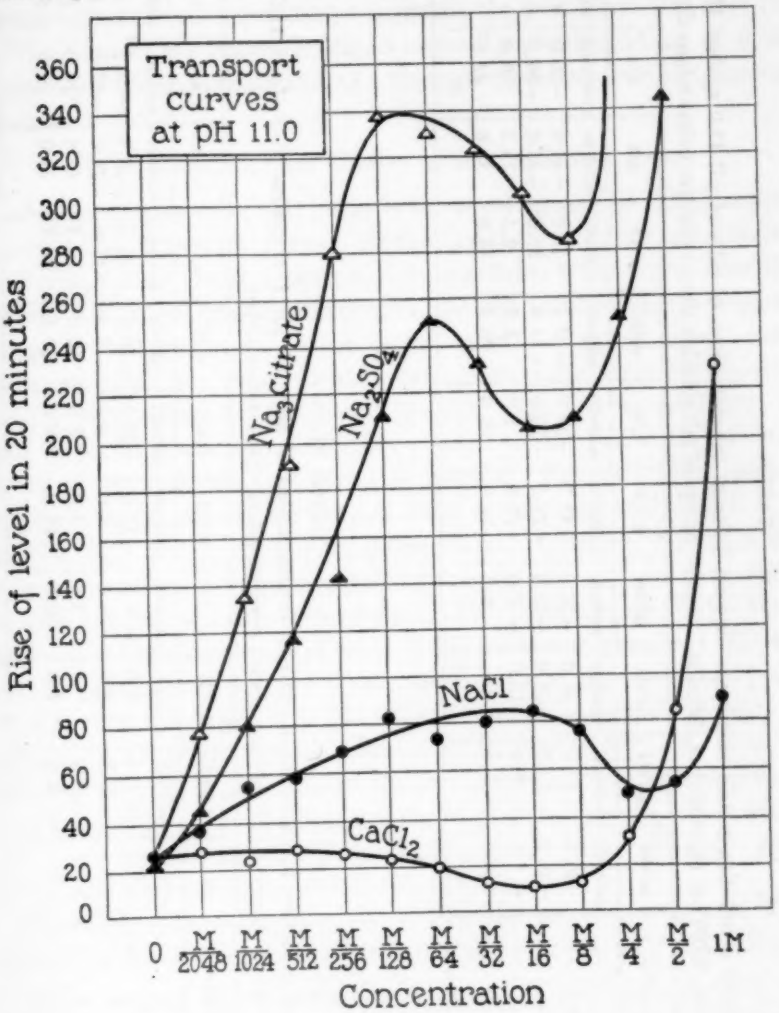

FIG. 3. Transport curves at pH 11.0. Notice reversal of relative efficiency of anions and cations between the curves in Figs. 1 and 3, due to the fact that the sign of charge of liquid inside the pores is positive at pH 3.0 and negative at pH 11.0.

TABLE V.

P.D. across a collodion-gelatin membrane between different concentrations of salts of pH 11.0 against H_2O of pH 11.0 (alkali used, KOH) *at beginning* of diffusion. Sign of charge of salt solution always positive unless minus sign is added.

Concentration.	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M
	millivolts	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
Na_3 citrate.....	−20.0	−27.0	−38.0	−34.0	−37.0	−39.0	−33.5	−37.0	−44.0	−46.0	−52.0	−56.0
Na_2SO_4.....	−15.0	−23.0	−25.0	−28.0	−29.0	−25.0	−23.0	−21.0	−23.0	−26.5	−30.0	
NaCl.....	− 5.0	− 8.0	−13.0	− 7.0	−10.0	1.0	4.5	9.5	13.5	19.0	22.0	24.0
$CaCl_2$.....	− 4.0	− 2.5	0	6.5	14.0	20.0	26.5	33.0	38.0	42.5	49.0	50.0

TABLE VI.

Diffusion potentials in millivolts of different concentrations of salts of pH 11.0 against H_2O of pH 11.0 (alkali used, KOH.) Sign of charge of salt solution always positive unless minus sign is added.

Concentration.	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2
	millivolts	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
Na_3 citrate.....	0	−4.0	−6.0	− 9.0	−12.0	−15.5	−20.0	−24.0	−28.5	−33.5	−38.0	−43.0
Na_2SO_4.....	0	−2.5	−3.0	− 4.0	− 5.0	− 6.5	− 8.5	−11.0	−14.0	−17.0	−19.5	−22.0
NaCl.....	0	1.0	1.5	3.0	5.0	8.0	10.0	12.5	15.0	18.0	20.5	23.5
$CaCl_2$.....	0	4.5	7.0	10.5	15.0	19.5	23.5	28.5	34.0	39.0	43.0	48.0

We can say that the sign and order of the values of the potentials are the same in Tables V and VI indicating that the P.D. across the membrane is essentially of the nature of a diffusion potential. The negative P.D. across the membrane in Table V is, however, always greater than the corresponding P.D. across the membrane in Table VI.

The drop in the curves in Fig. 3 beyond a concentration of M/64 is due again to the diminution of ϵ through the increase in the concentration of salts.

III. The Transport Curves at the Isoelectric Point.

The main purpose of this paper is the investigation of the transport curves at the isoelectric point. At this point the gelatin is not ionized and salts cannot cause a charge of the particles unless they alter the pH or cause the formation of complex protein salts which undergo an electrolytic dissociation. This latter seems to occur when salts with trivalent (or tetravalent?) cations or salts with tetravalent anions are added to isoelectric gelatin, since the addition of this kind of salts has a similar effect as the addition of acid or alkali respectively to isoelectric gelatin. No such effect seems, however, noticeable in the case of salts of the type of NaCl, $CaCl_2$, or Na_2SO_4. These latter salts influence the transport curves at the isoelectric point in approximately the same way as does cane-sugar or grape sugar; *i.e.*, only osmotically. The transport curves for these latter salts show no electrical effect at the isoelectric point but only the osmotic effect (Fig. 4). In these experiments at the isoelectric point special care was taken that the gelatin film of the membrane was at the isoelectric point at the beginning of the experiment; *i.e.*, that the pH was 4.7. The gelatin used for the film formation was isoelectric and in addition the collodion-gelatin bags were kept in water which had been brought to pH 4.7, by adding acetic acid. The salt solutions also were carefully brought to pH 4.7.

Table VII shows that the P.D. across the membrane is very high at pH 4.7.[8] If, therefore, at this pH there exists only a small P.D.

[8] If we compare the P.D. across the membrane for the NaCl and $CaCl_2$ and $CeCl_3$ solutions with the diffusion potentials (which are not given in this paper), it is seen that the P.D. across the membrane is greater than the diffusion potentials for the same solutions, as if the membrane retarded the motion of the cations. The same was found when the pH was 3.0. It is, therefore, obvious that this cation retention is not caused by the charge of the gelatin.

TABLE VII.

P.D. in millivolts across a collodion-gelatin membrane between different concentrations of salts of pH 4.7 (isoelectric point of gelatin) against H_2O of pH 4.7, *at beginning of experiment.* Sign of charge of solution always positive unless minus sign is added.

Concentration.	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16	M/8	M/4	M/2	1 M	2 M
	millivolts	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
KCl.....		1.0	0.5	− 1.0	− 3.5	− 2.5	− 1.0	− 1.0	− 0.5	0	0	0	0	0
NaCl.....	4.0	9.0	10.0	13.0	15.0	19.0	22.0	25.0	28.0	27.0	28.0	33.0	34.0	
LiCl.....	0	13.0	14.0	18.0	26.0	32.0	38.0	36.0	39.0	49.0	51.0	57.0	55.0	59.0
$MgCl_2$.....	4.0	20.0	25.0	35.0	81.0	48.0	52.0	55.0	62.5	64.0	74.0	72.5	75.0	
$CaCl_2$.....	5.0	27.0	31.5	33.0	39.0	45.0	44.0	52.0	54.0	62.5	68.0	70.0	71.0	
$BaCl_2$.....	3.0	14.0	23.0	25.0	27.5	33.0	43.0	48.0	52.0	57.0	57.0	61.0	64.0	
$MgSO_4$.....	1.5	1.0	4.0	6.0	8.0	10.0	11.0	10.0	9.0	10.0	9.5	9.0	5.0	
$Ce(NO_3)_3$.....	0	17.0	28.0	41.0	44.0	49.0	50.0	54.0	55.0					
Na_2SO_4.....	4.0	− 4.5	− 7.0	− 8.0	−11.0	−14.0	−16.0	−18.5	−20.0	−25.0	−28.0	−32.5		
$Na_4Fe(CN)$		−28.0	−30.0	−30.0	−31.0	−33.0	−35.0	−37.5	−45.0	−50.0	−60.0			
Cane-sugar.....	3.0	2.5	5.0	2.5	4.0	3.0	3.5	3.0	2.5	2.5	3.0	− 1.0	−1.5	

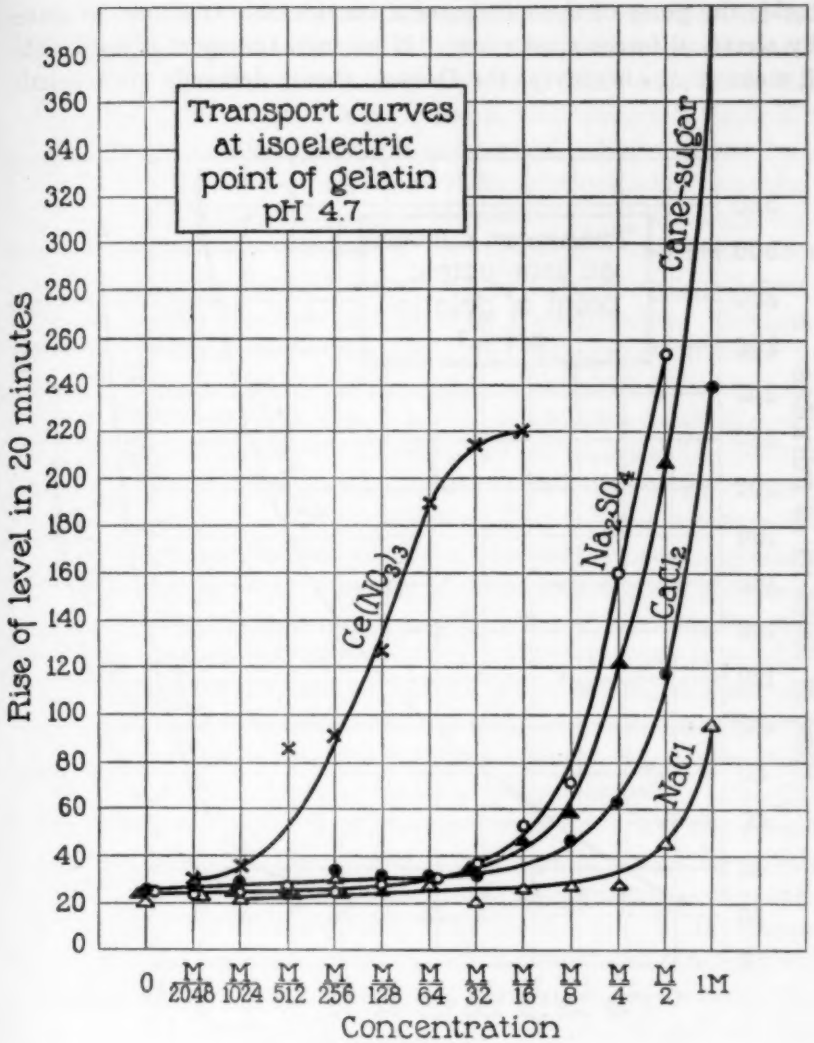

FIG. 4. Transport curves at the isoelectric point of gelatin. The curves for NaCl, $CaCl_2$, and Na_2SO_4 resemble the transport curve for cane-sugar showing that osmotic forces suffice to explain these curves. The curve for $Ce(NO_3)_3$ suggests that this salt confers a positive charge to the gelatin.

inside the pores of the membrane a considerable transport of water by electrical forces must occur. If no such transport is noticeable, it means that ϵ is zero (as the Donnan theory demands, since gelatin

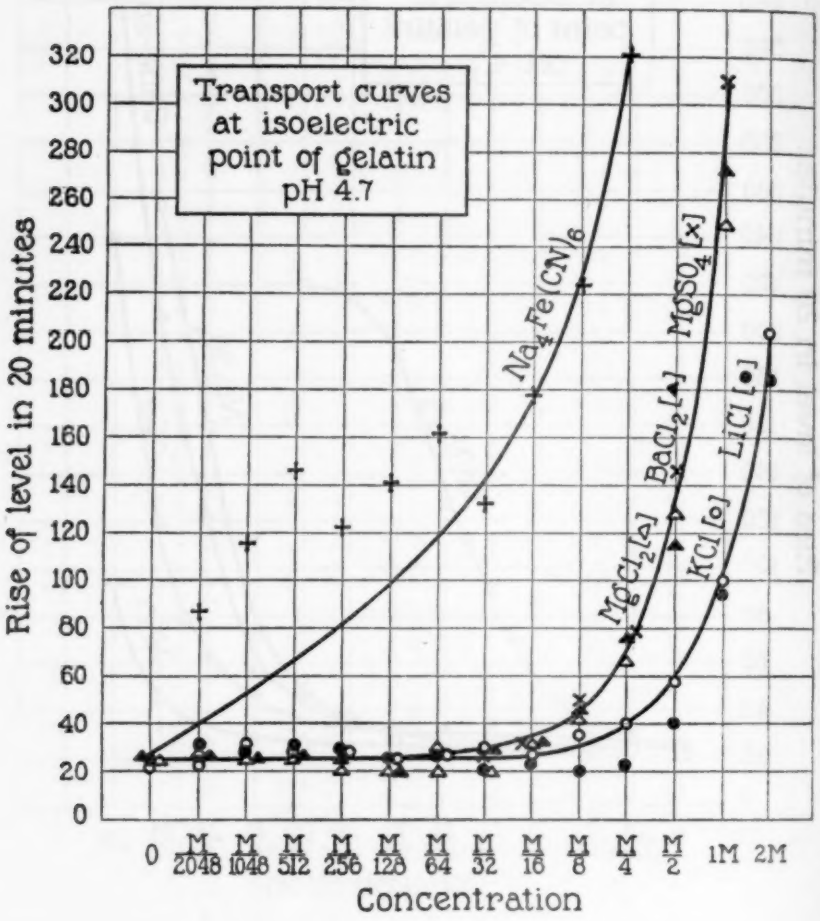

FIG. 5. Transport curves at the isoelectric point. Those for KCl, LiCl, $MgCl_2$, $BaCl_2$, and $MgSO_4$ resemble the transport curves for cane-sugar while $Na_4Fe(CN)_6$ seems to transfer a negative charge to the isoelectric gelatin.

is not ionized at its isoelectric point and does not combine with salt), and that there is no additional source of P.D. which might be ascribed to adsorption. Figs. 4 and 5 give the transport curves for cane-sugar, NaCl, KCl, LiCl, $MgCl_2$, $MgSO_4$, $BaCl_2$, $Ce(NO_3)_3$, and $Na_4Fe(CN)_6$ at pH 4.7; *i.e.*, when gelatin is non-ionized. There cannot be the least doubt that the curves for all these salts (with the exception of $Ce(NO_3)_3$ and $Na_4Fe(CN)_6$) are of the nature of the cane-sugar curve; *i.e.*, they show only that part of the curve which corresponds to the second rise of the transport curves in Figs. 1 and 3, and which must be ascribed chiefly, if not exclusively, to the osmotic forces. It might be added that the curve for Na_2 oxlate does not commence to rise until the concentration of the salt is M/16. The initial rise and drop of the transport curves in Figs. 1 and 3, which is the expression of the electrical forces, is entirely lacking in all the curves at the isoelectric point of gelatin, Figs. 4 and 5, with the exception of the curves for $Ce(NO_3)_3$ and $Na_4Fe(CN)_6$, to which we shall now give our attention.

In previous publications the writer has already called attention to the fact that on the alkaline side of the isoelectric point the presence of salts with a trivalent cation has the effect of reversing the sign of the P.D. between gelatin and water. When the pH is > 4.7, *i.e.* when gelatin exists in the form of Na gelatinate, the Donnan equilibrium causes the expulsion of NaOH from the gelatin into the bounding liquid with the result that gelatin assumes a negative and the bounding liquid a positive charge. When, however, some $CeCl_3$ or $LaCl_3$ is added the liquid assumes a negative and the gelatin a positive charge.[9] This reversal of the sign of charge by trivalent cations had been discovered by Perrin in his experiments on electrical endosmose.[10] The reversal may either be due to a reaction between $Ce(NO_3)_3$ and isoelectric gelatin, in which a compound is formed which dissociates into a complex positively charged gelatin-Ce cation and a negative ion, presumably NO_3, or the addition of the salt brings the pH to a value below 4.7. In either case the behavior of the curve for $Ce(NO_3)_3$ in Fig. 4 becomes clear. When $Ce(NO_3)_3$ solutions of pH 4.7 are separated by a collodion-gelatin membrane (of

[9] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 659.

[10] Perrin, J., *J. chim. physique*, 1904, ii, 601; 1905, iii, 50. Notice sur les titres et travaux scientifiques de M. Jean Perrin, Paris, 1918.

pH 4.7) from water of pH 4.7, the $Ce(NO_3)_3$ solution assumes a positive charge as is shown in Table VII. If now the Ce causes the liquid cylinders inside the pores to be charged negatively an electrical transport of water into the solution must occur commencing at a low concentration of the salt and in the way characteristic for the electrical transport curves in Figs. 1 and 3. The curve for $Na_4Fe(CN)_6$ in Fig. 5 suggests that $Na_4Fe(CN)_6$ causes solid isoelectric gelatin to assume a negative charge. The question is, whether this happens only in the case of trivalent (and probably tetravalent) cations and tetravalent anions. It is obvious that all the transport curves for salts with divalent cations, Mg, Ca, and Ba, in Figs. 4 and 5, commence to rise at a slightly lower concentration than the transport curves for the salts with monovalent cation, KCl, NaCl, and LiCl. It might be argued that the salts with bivalent cation transfer also a positive charge to isoelectric gelatin at a concentration above M/8 and that the same is true for the salts with monovalent cations, the difference being that the concentration of the salt required for this effect is very high for salts with monovalent cation, slightly lower for salts with bivalent cation, and very low for salts with trivalent cation.

On the other hand it should be pointed out that the transport curve for cane-sugar rises even more rapidly than that for $CaCl_2$ so that there is in reality no need to assume that the $CaCl_2$ charges solid isoelectric gelatin positively. The difference between the transport curves for NaCl and $CaCl_2$ in Fig. 4 is no greater than the difference between the transport curves for cane-sugar and grape sugar. As far as the experiments on anomalous osmosis are concerned, there is no reason to assume that the salts with divalent cations or monovalent cations transfer a positive charge to the isoelectric gelatin or to gelatin of any pH or that the bivalent anions transfer a negative charge, though it is not absolutely disproved that this may not happen at high concentrations of the salts.

It was thus far left undecided whether the positive electrification of isoelectric gelatin by $Ce(NO_3)_3$ in Fig. 4 was due to a change of the pH so that the gelatin was no longer isoelectric or to the formation of a salt between isoelectric gelatin and $Ce(NO_3)_3$ dissociating into a positively charged complex gelatin-Ce ion and negatively charged NO_3 ions. To settle this question experiments were made with

buffer solutions consisting of mixtures of M/50 acetic acid and M/50 Na acetate in the proper proportions to give a pH 4.7, 5.6, or 3.4. Solutions of different concentrations of $Ce(NO_3)_3$ were made up in these buffer solutions and put into the collodion bags. The outside solutions

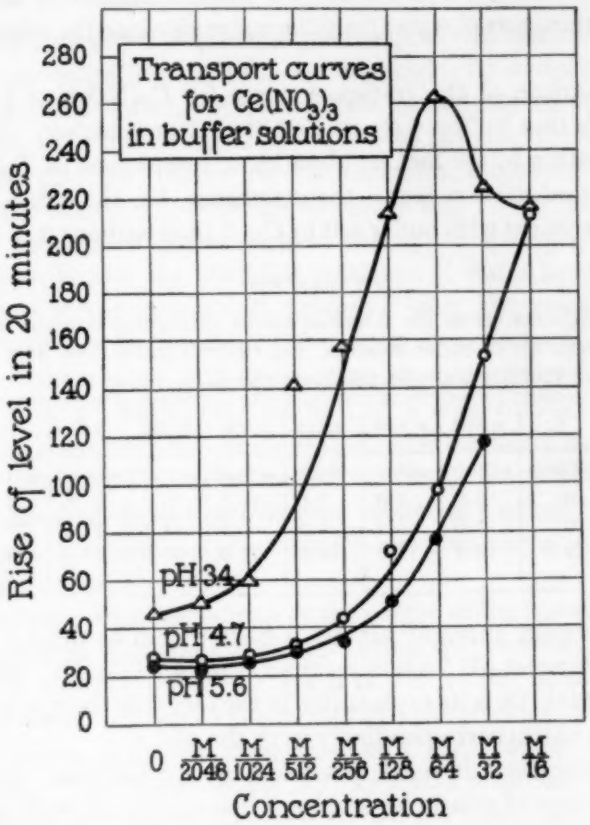

FIG. 6. Influence of $Ce(NO_3)_3$ on the transport curve in the presence of buffer solutions (mixtures of M/50 acetic acid and M/50 Na acetate).

were the same buffer solutions without the $Ce(NO_3)_3$. The curves in Fig. 6 represent the transport values in 20 minutes. We will first direct our attention to the transport curve at pH 4.7. It is clear that at the isoelectric point the transport curve for $Ce(NO_3)_3$ rises again steeply, thus supporting the idea that the $Ce(NO_3)_3$ reacts with the

protein in the way suggested, thereby giving rise to a double layer in the pores in which the gelatin is positively and the liquid negatively charged. Table VIII shows that at pH 4.7 there is a high P.D. across the membrane in which the $Ce(NO_3)_3$ solution assumes a positive charge. If now the liquid inside the pores is negatively charged an electrical transport of water from the water side into the solution must occur.

A comparison of the transport curve for $Ce(NO_3)_3$ of pH 4.7 in Fig. 6 with that in Fig. 4 shows that the latter is higher. This finds its explanation in the fact (evident by a comparison of Tables VIII and VII) that the P.D. across the membrane, *i.e.* the value E, is less in the experiment with buffer salt in Fig. 6 than without it in Fig. 4.

TABLE VIII.

P.D. in millivolts across the collodion-gelatin membrane between solutions of $Ce(NO_3)_3$ made up in buffer solution, and buffer solution free from $Ce(NO_3)_3$. The $Ce(NO_3)_3$ solution is always positively charged.

Concentration of $Ce(NO_3)_3$.	0	M/2048	M/1024	M/512	M/256	M/128	M/64	M/32	M/16
pH	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*	*millivolts*
4.7	0	2.0	4.0	7.0	11.5	16.0	21.0	27.0	34.0
5.6	0	1.0	2.5	4.5	7.5	11.5	17.0	23.0	28.0
3.4	2.0	10.5	17.5	26.0	29.0	32.0	35.0	38.0	44.0

The transport curve at pH 3.4 is higher than at pH 4.7 and the transport curve at pH 5.6 is lower than at pH 4.7 (Fig. 6). Table VIII shows that this finds its explanation in the fact that the P.D. across the membrane varies correspondingly with the pH.

As a consequence it seems possible that the $Ce(NO_3)_3$ causes the positive charge of gelatin by forming a dissociable salt with gelatin in which the positive ion is a complex gelatin-Ce ion. In other words, the salt reacts with isoelectric gelatin in a similar way as acid does. If this assumption is correct the P.D. between gelatin and bounding $Ce(NO_3)_3$ solution must be ascribed to a Donnan equilibrium, in which the $Ce(NO_3)_3$ plays a similar rôle as the HCl.

The difference between gelatin chloride and the hypothetical gelatin-$Ce(NO_3)_3$ salt is this that it is much easier to remove by washing the $Ce(NO_3)_3$ from powdered gelatin than it is to remove the HCl.

In an analogous way we must assume that isoelectric gelatin can combine loosely with $Na_4Fe(CN)_6$ whereby negatively charged complex gelatin-$Fe(CN)_6$ ions and positively charged Na ions are formed.

SUMMARY AND CONCLUSIONS.

1. It has been shown in previous publications that when solutions of different concentrations of salts are separated by collodion-gelatin membranes from water, electrical forces participate in addition to osmotic forces in the transport of water from the side of the water to that of the solution. When the hydrogen ion concentration of the salt solution and of the water on the other side of the membrane is the same and if both are on the acid side of the isoelectric point of gelatin (*e.g.* pH 3.0), the electrical transport of water increases with the valency of the cation and inversely with the valency of the anion of the salt in solution. Moreover, the electrical transport of water increases at first with increasing concentration of the solution until a maximum is reached at a concentration of about M/32, when upon further increase of the concentration of the salt solution the transport diminishes until a concentration of about M/4 is reached, when a second rise begins, which is exclusively or preeminently the expression of osmotic forces and therefore needs no further discussion.

2. It is shown that the increase in the height of the transport curves with increase in the valency of the cation and inversely with the increase in the valency of the anion is due to the influence of the salt on the P.D. (E) across the membrane, the positive charge of the solution increasing in the same way with the valency of the ions mentioned. This effect on the P.D. increases with increasing concentration of the solution and is partly, if not essentially, the result of diffusion potentials.

3. The drop in the transport curves is, however, due to the influence of the salts on the P.D. (ϵ) between the liquid inside the pores of the gelatin membrane and the gelatin walls of the pores. According to the Donnan equilibrium the liquid inside the pores must be negatively charged at pH 3.0 and this charge is diminished the higher the concentration of the salt. Since the electrical transport is in proportion to the product of $E \times \epsilon$ and since the augmenting action of

the salt on E begins at lower concentrations than the depressing action on ϵ, it follows that the electrical transport of water must at first rise with increasing concentration of the salt and then drop.

4. If the Donnan equilibrium is the sole cause for the P.D. (ϵ) between solid gelatin and watery solution the transport of water through collodion-gelatin membranes from water to salt solution should be determined purely by osmotic forces when water, gelatin, and salt solution have the hydrogen ion concentration of the isoelectric point of gelatin (pH = 4.7). It is shown that this is practically the case when solutions of LiCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, $BaCl_2$, Na_2SO_4, $MgSO_4$ are separated by collodion-gelatin membranes from water; that, however, when the salt has a trivalent (or tetravalent?) cation or a tetravalent anion a P.D. between solid isoelectric gelatin and water is produced in which the wall assumes the sign of charge of the polyvalent ion.

5. It is suggested that the salts with trivalent cation, *e.g.* $Ce(NO_3)_3$, form loose compounds with isoelectric gelatin which dissociate electrolytically into positively charged complex gelatin-Ce ions and negatively charged NO_3 ions, and that the salts of $Na_4Fe(CN)_6$ form loose compounds with isoelectric gelatin which dissociate electrolytically into negatively charged complex gelatin-$Fe(CN)_6$ ions and positively charged Na ions. The Donnan equilibrium resulting from this ionization would in that case be the cause of the charge of the membrane.